Application of Chosen Plant Extracts in Fresh-Cut Apple for Substitution of Sulfur Dioxide

BY

MR. JATURONG AMONCHAISUP

ID.501-2712

A special project submitted to the School of Biotechnology, Assumption University in part fulfillment of the requirement for the degree of Bachelor of Science in Biotechnology

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Title	**:**	**Application of chosen plant extracts in Fresh-cut apple for substitution of sulfur dioxide**
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Dipl. oec. troph. Birgit Wessels
(Advisor)

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Jaturong Amonchaisup

ABSTRACT

The effect of plant extracts (clove and lemon balm extracts) on the inhibition of enzymatic browning has been investigated in order to compared with the use of sodium bisulfite. Also, the effect of extract's concentration, temperature, and pH has been studied. In order to observe the inhibition effect, fresh sliced apples were dipped in extract solution or sodium bisulfite solution for 10 min and then exposed to air at room temperature for 4 hours before measurement. The enzymatic browning of samples was evaluated by color measurement in CIELAB system using a colorimeter (Minolta CR-400, Japan). Total color difference (ΔE^*) and relative total color difference ($\Delta(\Delta E^*)$) were used as the indices of enzymatic browning inhibition in which a higher ΔE^* and lower $\Delta(\Delta E^*)$ values indicate a better inhibition. The results found that the optimization of clove extract provided a greater effectiveness than that of lemon balm extract on the inhibition of enzymatic browning. Although only the optimization of clove extract ($\Delta E^* = 1.51$ and $\Delta(\Delta E^*) = 4.27$) can provide a greater inhibitory effect than sodium bisulfite ($\Delta E^* = 1.81$ and $\Delta(\Delta E^*) = 4.18$), its use for sodium bisulfite substitution cannot be confirmed due to non-corresponding results of visual appearance. However, the optimization of clove extract still provided the considerable inhibitory effect on the enzymatic browning. It can be successively used as one alternative for natural antibrowning agents.

CONTENT

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NOMENCLATURE

Symbols

Symbols	Meaning
L*	Lightness coordinate
a*	Redness (+a*)/ Greenness (-a*) coordinate
b*	Yellowness (+b*)/ blueness (-b*) coordinate
ΔL*	Difference in L* value
Δa*	Difference in a*value
Δb*	Difference in b*value
ΔE*	Total color difference
$\Delta E_{\text{water control}}$	Total color difference of sample treated with water
Δ(ΔE*)	Relative total color difference (total color difference compared with water)

1. Introduction

As worldwide consumers' awareness on health issues has increased, there is a rising in the consumption of fruits and vegetables during the last years. This group of food is considered as a main source of vitamins and minerals, and also provides high nutritional value including antioxidant and free-radical scavenging properties [7]. Furthermore, a use of synthetic compounds for food preservation or quality improvement becomes a critical issue since consumers are more and more afraid of adverse health effects of these compounds [42]. Due to these circumstances, the food, which can highly maintain its natural quality and also provides conveniences to get along with modern life-style, is increasingly demanded.

As a result, a food industry has developed new processing techniques to produce minimally processed fruits and vegetables in order to meet those demands. Thus, the production and consumption of fresh-cut fruit and vegetables is rapidly growing and expanding during recent years [7, 51]. Among fruits, apples are the most popular cultivar consumed all over the world due to high nutritional value and availability. The consumption of apples is related to the prevention of different diseases such as cancer and heart disease [77]. Nowadays, fresh-cut apples have highly emerged as a popular fruit product in many sectors [55]. However, it is well known that the fresh-cut apples are more perishable and more susceptible to quality deterioration than original unprocessed raw materials [81].

A major problem for fresh-cut apple manufacturers is to retain the acceptable quality attributes of products and maintain its shelf-life until consumption. This is challenging for fresh-cut fruit industry since the tissue damage can occur through mechanical injury during harvesting, post harvest storage or processing. The latter is considered as a main step of quality deterioration [51]. Processing like peeling, cutting, coring and shredding leads to the cellular decompartmentalization and delocalization of enzymes and substrates. Consequently, there are undesirable quality changes in an apple tissue such as an increase in respiration rate, acceleration in ripening and senescence, microbial spoilage and biochemical deterioration (browning, off flavor, nutrient degradation and texture breakdown) [7, 51, 81]. Among the biochemical changes, the enzymatic browning has been figured out to be a major concern since the discoloration of apple slices has a main impact on the loss of aesthetic and nutritional quality as well as on the

market value for consumer acceptability and purchase decision [42, 89]. Discoloration is caused by two successive steps. It is initialized by a polyphenol oxidase (PPO) catalyzed reaction followed by various non-enzymactic reactions which lead to the formation of brown pigments (melanin) [6]. PPO is particularly present in mushroom, banana, potato, and apple tissue, so that these foods are more susceptible towards the enzymatic browning reaction [3, 63].

The most widespread method for controlling the enzymatic browning is the sulfurization [63]. Sulfites are successfully used as antibrowning agent which also possesses antioxidant and antimicrobial activity as well as being economical [55]. However, sulfites are banned by FDA for the use in fresh fruits and vegetables because of hazardous negative health impacts especially in asthmatic patients [56]. Besides, sulfites can lead to changes in the taste of the treated product [63]. Due to these reasons, alternative antibrowning agents respectively methods are needed in order to replace sulfites.

There are extensive researches on non-sulfite antibrowning agents, for instance, ascorbic acid, citric acid, thiol containing compounds, 4-hexyl resorcinol (4HR), etc. Moreover, there are different methods studied in order to control the enzymatic browning such as heat treatment, irradiation, high pressure treatment, oxygen exclusion, modified atmosphere packaging, etc [25]. But no other chemical agent respectively method is able to replace the use of sulfites sufficiently because they cannot meet all of the following criteria: inhibitory effect comparable to sulfite, practical, safe, consistent, economical and not affecting sensory food quality [29, 63].

Because of safety, price and potential activity of plant derived substances, it is possible to use plant extracts as the alternative antibrowning agent. Plant extracts contain the significant amounts of bioactive polyphenols possessing an antioxidant activity. This property could make the extracts serving as the effective antibrowning agent. Furthermore, the use of plant extracts as a substitute for sulfites may also have health promoting effects because their consumption is related to the risk reduction of different diseases [2, 9, 55, 63]. However, there are only a small number of investigations on antibrowning properties of plant extracts. Extensive studies on the antibrowning effect of plant extracts are needed to discover the potential as sulfite substitutes.

2. Objectives

1. To study the effect of extract concentration, temperature and pH of clove and lemon balm extract on the inhibition of the enzymatic browning of fresh-cut apples.
2. To study the interactive effect between extract concentration, temperature and pH on the inhibition of the enzymatic browning of fresh-cut apples.
3. To optimize the combination of the studied factors including: concentration, temperature and pH of clove and lemon balm extract, and compare their optimum responses on the inhibition of the enzymatic browning of fresh-cut apples.
4. To compare between the extracts at the optimum factor combination and sulfite on the inhibition of the enzymatic browning of fresh-cut apples.

3. Theoretical background

3.1 Production of fresh-cut apples

Currently, the worldwide consumption of fruit and vegetable products has highly increased because of a consumers' awareness on health issues [89]. Fruits and vegetables are rich in nutritional value especially vitamins which can help to promote healthiness and reduce the risk of diseases [7, 44]. However, these beneficial nutrients can be reduced or depleted during storage and processing [44]. Consumers are also afraid of the negative health effects of synthetic additives in food [42]. Furthermore, the lifestyle of the consumers has changed during the last years and a time-consuming preparation of food is considered to be inconvenient [75]. Prepared food especially fruits and vegetables thus became more popular [75]. The desired fruit and vegetable products should conform with the following attributes: they should show a constant quality including nutritional value, as well as sensorial quality. Besides, they should contain a minimum of chemical additives, and provide conveniences. To meet this demand, the food industry has focused on developing new techniques to produce minimally processed or fresh-cut fruits and vegetables.

Apples are among the most popular fruit grown in the world [97]. A global apple production is regarded as approximately 12% of a total fruit production [28]. Minimally processed or fresh-cut apples are highly consumed worldwide because of high availability and health benefits. In term of nutrition, apples contain a significant amount of fibers, vitamins and flavonoids with providing few calories but no fat, cholesterol or sodium [10, 33, 97]. Based on the apple popularity, Jonagold is one of cultivars that is widely planted throughout Europe [12]. This cultivar has an excellent quality and is suitable for a fresh-food market and processing [12]. Because of the slow browning reaction observed in Jonagold, there is a suggestion that Jonagold could be a good candidate for minimal processing [56].

3.1.1 Quality parameter of Fresh-cut apples

The quality of fresh-cut fruits is contributed by the combination of attributes, properties, or characteristics that determine their value to consumers, and the consumers' purchasing decision [47]. The quality of fresh-cut apples can be grouped into three major quality factors: appearance, texture, and microbiological factors [23, 88].

1. Appearance

An appearance has a major influence on a quality perception and seems to be the most important factor. The appearance factors of apples or fresh-cut apple include size, shape, color, gloss, and freedom from defects and decays [47].

Color and size are two very important quality criteria for commercial apples [79]. But color is the most influential to the quality evaluation of apples and thus fresh-cut apples [23]. Color is one of factors affecting the apples' appearance. It is also used as an index to determine the physiological changes of apples during growth, development, and damage [22]. Besides, it is used within the quality control department as a significant parameter to assure the quality of apple during processing until purchasing. For fresh-cut apples, the consumers put the major consideration of color on the internal flesh. The damage and bruising developed during processing and storage under unsuitable conditions is a cause of the enzymatic browning of fruit tissues [22]. Subsequently, the enzymatic browning on apple flesh is a major cause of color deterioration which is an unacceptable characteristic of apple slices. This defect convinces the consumer rejection on buying flesh-cut apples. Color control is one way to improve color quality and provide apples or fresh-cut apples with a desirable appearance to consumers.

For size selection, most of consumers prefer the big size of apples with uniformly circular shape in which when they are processed to fresh-cuts, the desirable shape and size of apple slices are obtained [22].

2. Texture

The texture of apples can be described by following related terms: firmness, crispness, juiciness, fibrousness, toughness, and softness [8, 88]. Textural characteristic of fruits is not only necessary for eating and cooking quality but also shipping ability [47]. Normally, crisp, firm and juicy tissues are desirable in apple flesh but the development of excessive toughness is not acceptable. Some degree of softening is also required in apple flesh but over-softening is not desirable and is the sign of senescence or internal decay [44]. Normally, the loss of desirable texture is related with the effect of aging and processing. During prolonged storage of apples, softening of fruit, decreasing in crispness and acid/sugar ration, and loss in cell wall integrity are obtained. During mechanical operation, the cut surfaces of apple slices are damaged so that enzymes are released. Accordingly, it causes a degradation of parenchyma tissue and cell wall. However, the loss of texture can be prevented by calcium treatment.

3. Microbiological spoilage

Microbiological spoilage is another major factor affecting the quality of apples and fresh-cut apples inasmuch as microbial growth can lead to the loss of good appearance, flavor, texture and nutritional quality. During processing, the natural protection of fruit is generally removed thus minimally processed fruits are generally more perishable to microbial growth than the whole intact raw materials [67]. A wide variety of microorganisms including mesophilic bacteria, lactic acid bacteria, fecal coliforms, yeast, and filamentous fungi are actively growing on fresh-cut apples [47, 73]. Therefore, the minimally processing may increase the incident of microbial spoilage as well as the consumers risk to acquire the diseases caused by pathogenic flora [76]. Besides, the bacterial infection can also promote browning of apples. The most potent microorganism causing spoilage in apples are fungi since the acid condition of apple suppresses the growth of bacteria [44]. However, washing and antimicrobial treatment like chlorination are applied as a preventive method of a microbial contamination.

3.1.2 Production of fresh-cut apples

The industrial production of fresh-cut apples has involved with many processing steps as described in the following steps.

Step 1 Receiving raw materials: Whole fresh apples are unloaded and transferred to the processing line by the staff [47].

Step 2 Cleaning and Peeling: Incoming whole apples are washed with water to remove dirt, mud or organic materials. Apples are then sent to the peeling machine for peel removal [47].

Step 3 Coring and slicing: Apples are transferred into the coring machine to remove the stems, blossom cavities and seeds. Finally, apples are cut into slices at size and shape ordered by clients.

Step 4 Visual Inspection: A visual inspection is continuously taken place to determine the quality of apple slices whether they are fresh, clean, free from defects or foreign materials, and intact in size and shape, etc [41].

Step 5 Correction: After inspection, if apple slices do not conform to the specification, the corrective action is taken by the manager of QA department [41].

Step 6 Packing and inspection: Fresh-cuts are packed into plastic bags. Meanwhile, the visual inspection is secondarily taken place to check the quality of the products.

Step 7 Correction: After inspection, foreign materials are sorted out from apple slices. In case of an agglomeration of foreign bodies, corrective action is taken by the manager of QA department to improve the process [41].

Step 8 Sealing and labeling: After passing the inspection, the plastic bags are sealed and labeled with product name, production date and, manufacturer.

Step 9 Storage: Finally, the bags of apple slices are transferred and stored in the storage room at 4-5°C before distribution to retail shops or supermarkets.

The processing temperature in all steps is controlled to around 4-5 °C in order to maintain the quality of the products [13].

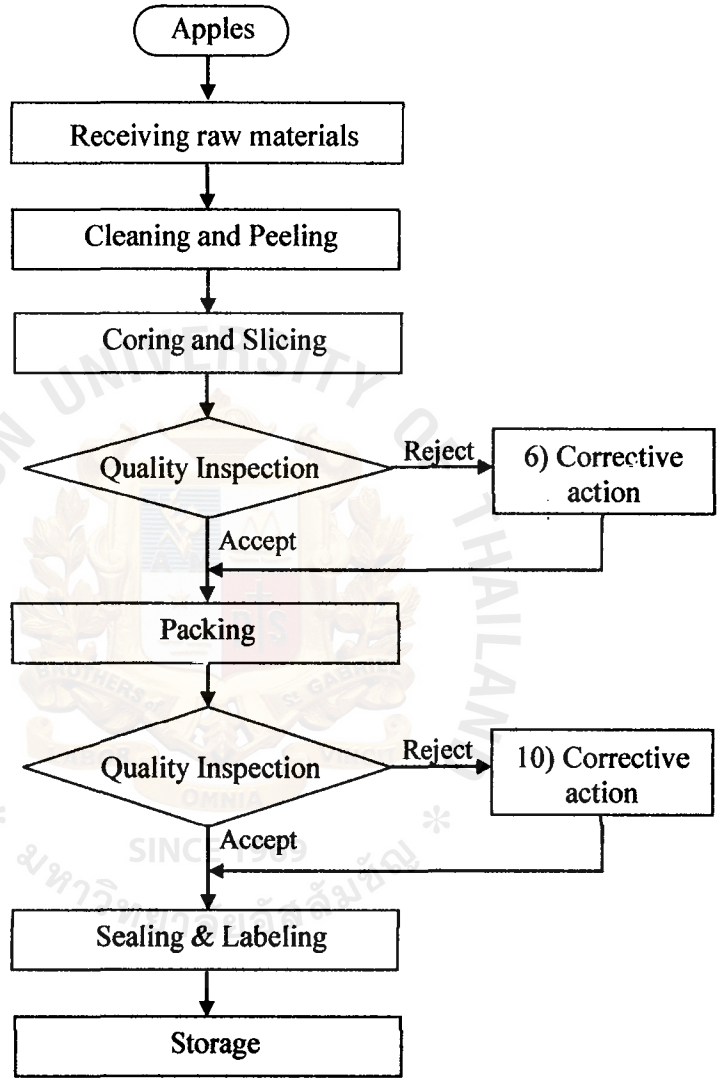

Figure 3.1: Flowchart of fresh-cut apples production (*Meyer Gemüsebearbeitung GmbH, Germany*).

Because of processing steps especially slicing and coring, the mechanical injury and tissue damage have been caused on the cut surface of apple slices. Consequently, physiological and biochemical changes such as an increase in respiration rate, reduction of texture firmness,

acceleration of senescence, microbial growth and enzymatic browning can taken place leading to quality loss. But a major concern is put on the enzymatic browning because this reaction is taken place instantly during the processing, and the outcome of the reaction is noticeably observed. This reaction highly deteriorates the appearance quality especially color parameter which is the most important attribute of fresh-cut apples.

3.2 Enzymatic browning

An enzymatic browning is a discoloration process occurring in fruits, vegetables, and marine animals. It is caused mainly by the action of a group of enzymes called polyphenol oxidases (PPO) which act on phenolic compounds to form quinones that further change to brown pigments via the non-enzymatic steps [31]. These enzymes are particularly present in mushroom, banana, potato, peach and apple. This reaction in fruits and vegetables can occur as a result of plant senescence, mechanical injuries, and pathogen infection [94]. But enzymatic browning of fresh cut fruits and vegetables is usually resulting from mechanical injuries during processing as mentioned before. The enzymatic browning causes a flavor alteration, loss of nutritional value and reduced shelf-life of fruits [99]. For this reason, it leads to the economical loss of over 50% of fruit crop especially tropical and subtropical varieties [53]. It is thus recognized as one of major problems in the fruit industry. However, the brown discoloration is desirable in some food products such as raisins, prunes, coffee, tea, cocoa, etc since it improves the appearance and flavor qualities of these products. To initiate the reaction, it requires the presence of three major components: phenolic substrates, enzymes (mainly PPO), and oxygen.

3.2.1 Process of enzymatic browning

The principle of enzymatic browning is due to the action of the enzymes. Normally, there are a number of enzymes responsible for browning but the major metabolic enzymes are peroxidases (POD) and polyphenol oxidases (PPO) [1].

3.2.1.1 Peroxidases

Peroxidases (EC 1.11.1.7) belongs to the group of oxidoreductases. Its primary function is to catalyze the decomposition of hydrogen peroxide (H_2O_2) in the presence of hydrogen donors [47]. But its contribution on browning is owing to their ability involved in the oxidation of polyphenols such as catechins, hydroxycinnamic acid derivatives, and flavans [47]. The primary products of the phenolic oxidation are quinones which undergo further reactions to form brown pigments [66]. The pH optimum of POD varies in accordance with the enzyme source but the pH optimum in fruits is generally ranged from 4.0 to 6.5 [68]. However, POD commonly appears to be less involved in enzymatic browning in fruit and vegetables because the content of H_2O_2 substrate is very low in the plant tissue.

3.2.2.2 Polyphenol oxidases

Unlike POD, polyphenol oxidases (PPO) is the enzyme that is more predominant for the enzymatic browning of fruits. This enzyme also belongs to the group of oxidoreductases acting on phenolic compounds (substrate) [30]. It is ubiquitous and seems to widely distribute in microorganisms, plants, and animals [94]. This enzyme is present in almost ail the plants, and they are most abundant or active in fruits such as apples as mentioned before [68]. However, it is first discovered in mushroom [30]. Due to PPO has a broad substrate specificity, it is also reffered to as tyrosinase, phenolase, monophenol oxidase, diphenol oxidases, catecholase, catechol oxidase, cresolase [29, 105]. PPO catalyzes the oxidation of phenolic compounds at the expense of oxygen as a co-substrate to produce quinones as a product [30, 53]. It is important to study the characteristic, function and mechanism reaction of PPO to get a profound understanding of the enzymatic browning so that the potential inhibition methods can be proposed.

Physiological properties

PPO can be characterized in term of a distribution and location, structure, and substrate specificity. Regarding location, it appears to reside in the mitochondria and chloroplasts but it is only released to cytoplasm in a soluble form upon wounding, senescence, or organelle degeneration [30].

The substrate specificity of plant PPO is broadly different in accordance with the source of enzyme. In apples, the common phenolic substrates are chlorogenic acid , catechin, catechol, caffeic acid, DOPA, p-cresol, leucocyanidin, flavonol glycosides, and 3,4-dihydroxy benzoic acid [53]. But PPO in apples preferentially acts on chlorogenic acid since this phenolic compound is present in apples at higher concentration than the others [68]. As chlorogenic acid is the most prevalent in flesh, the brown discoloration obviously appears on the apple flesh.

In uncut or undamaged fruits, phenolic substrates are separated from PPO enzyme by cellular compartmentalization [63]. But when a cell membrane is damaged through processing, there is the decompartmentalization of cellular structure [93]. Therefore, phenolic compounds, PPO and O_2 can come into contact to each others to initiate the enzymatic browning reaction [53, 94].

Reaction mechanisms

PPO has been involved to catalyze two basic reactions. Firstly, it catalyzes the hydroxylation of monophenol into diphenol. Secondly, it also catalyzes the oxidation of diphenol to colored quinones which could further lead to the production of brown pigment, melanin. In spite of extensive studies on PPO, the complete reaction mechanism is still unclear due to the complicated reactions involved. Normally, the polyphenol oxidases is present in many forms and each enzyme is responsible for catalyzing a number of reactions [40]. PPO or tyrosinase can be categorized into three forms: oxytyrosinase (oxy), deoxytyrosinase (deoxy) and mettyrosinase (met) form as shown in the figure 3.2 [106]. These three forms are distinguished from each other based on the different configuration concerning copper molecules which are the prosthetic group of the enzyme [99, 106]. The clarification of each form is stated below.

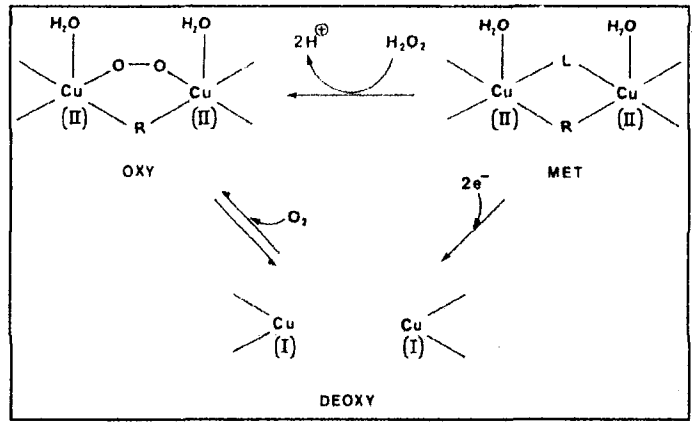

Figure 3.2: Different form of polyphenol oxidases [106].

For the oxy form, the two cupric (Cu (II)) molecules are bound with protein residues (usually histidines) in the equatorial plane and bound with ligands such as H_2O in the axial plane [106]. These two coppers are also bridged by endogenous protein ligand represented as -R- (figure 3.2) and exogenous oxygen molecule bound as peroxide [106, 78]. In the met form, coppers ions are bridged by other exogenous ligand such as NO_2^-, N_3^- instead of peroxide [106, 78]. Last, the deoxy form of enzyme only contains a bicuprous (Cu (I)) structure without bridging [106].

These three forms have been involved in two initial steps of enzymatic browning reaction which are hydroxylation of substrates (mainly monophenols) and oxidation of formed diphenols.

Reaction mechanisms: Hydroxylation and oxidation of monophenol

The reaction pathway for initial step of enzymatic browning can be divided into two categories whether the substrates are the monophenols or diphenols. If the starting substrate is monophenols, the reaction will run according to the pathway illustrated in figure 3.3. Firstly, the enzyme is in the deoxy form (I) which further bind with molecular oxygen to give the oxy form (II). Then, the oxy form (II) binds with monophenol substrate to give the O_2-PPO-monophenol complex (III) where the hydroxyl group of the monophenol has replaced one of H_2O molecule on one of the Cu (I) (form III). Form (III) subsequently undergoes an arrangement to form the trigonal bipyramidal intermediate (IV). This rearrangement of the copper coordination geometry

iabilizes the peroxide from one copper while leaving the reactive peroxide that can hydroxylate the phenol substrate. Consequently, the met form is produced whose the exogenous ligand is *o*-diphenol substrate (form V). The *o*-diphenol then undergoes the oxidation into *o*-quinone (VI) dissociated from the enzyme while the two Cu (II) are reduced to Cu (I) resulting in the formation of deoxy PPO (form I). This deoxy form can recycle in the process again [30, 78, 106].

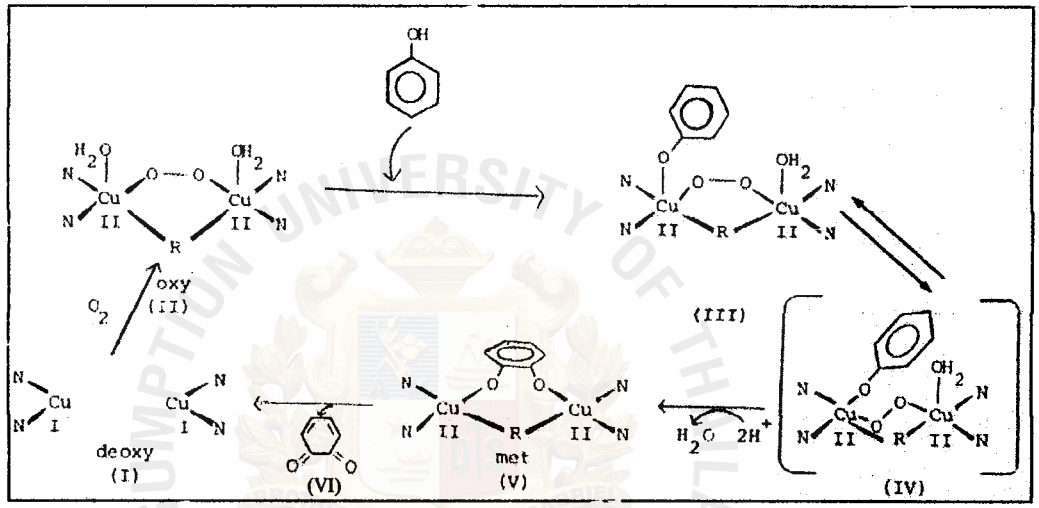

Figure 3.3: Mechanism of hydroxylation and oxidation of monophenol [78].

Reaction mechanisms: Oxidation of diphenol

Although the oxidation of diphenols has been previously described in above paragraph, that explanation is associated with the reaction whose starting substrates are monophenols. But if the starting substrates are diphenols, the oxidation of these diphenols will use different pathway as illustrated in figure 3.4. In the oxidation of diphenol, the phenolic and oxygens are able to coordinate with the enzyme in all of three forms but the most common coordination occurs with the oxy form. For the met form, o-diphenol can bind with the met form, followed by the reduction of two Cu (II) into bicuprous ion (form I). Consequently, the deoxytyrosinase is formed while *o*-diphenol substrate is oxidized into *o*-quinone liberated from enzyme [106].

Deoxy form can further be oxygenated to give the oxy form. This oxy form then binds with o-diphenol via the oxygen atom of the two hydroxyl groups of *o*-diphenol resulting in the formation of O_2-PPO-diphenol complex (form II). Subsequently, *o*-diphenol is oxidized to *o*-quinone while the enzyme is reduced to the met form [30, 106].

Alternatively, before oxygenation, the deoxy form can coordinate with *o*-diphenol to form the coordinated complex (form III). This complex further binds with oxygen molecule to form O_2-PPO-diphenol complex (form II). Finally, *o*-diphenol is oxidized to *o*-quinone with the enzyme reduction met form [106].

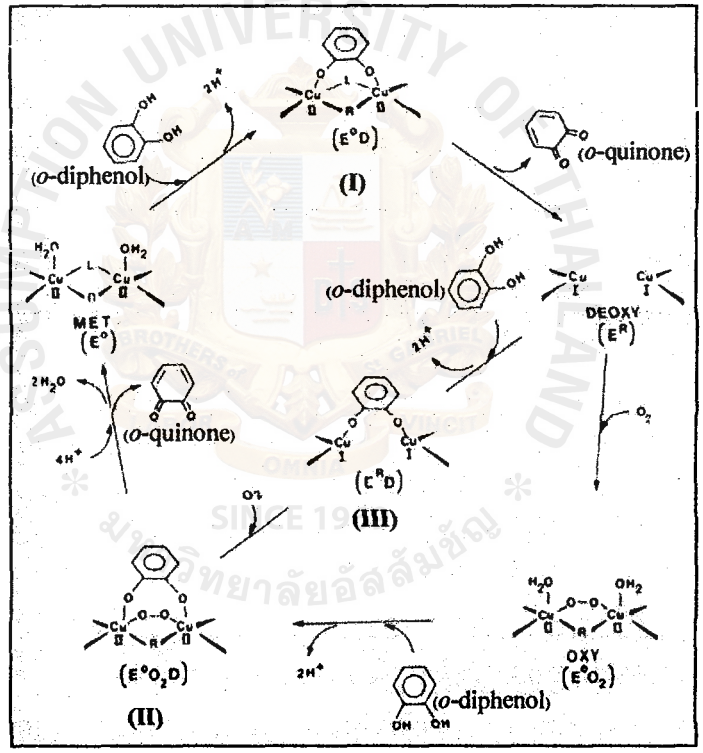

Figure 3.4: Catalytic cycle for oxidation of *o*-diphenol substrate to *o*-quinone [106].

From the overall reactions, it can be summarized that PPO is able to catalyze two distinct reactions which are the initial steps of the enzymatic browning. Firstly, it catalyzes the hydroxylation of monophenolic substrate into *o*-diphenolic compounds [68]. This reaction is

referred as cresolase activity [37]. Secondly, it also catalyzes the oxidation of the *o*-diphenols into the corresponding quinones [106]. This reaction is defined as catecholase activity [105]. The catecholase activity is always present in all plant PPO, but the cresolase activity is not [66]. Moreover, catecholase activity is more affecting on the enzymatic browning of food than cresolase activity because of a higher catalytic rate, the abundance of dihydroxphenols in food, and its association in production of brown pigment [37, 68].

Polyphenol oxidases isozymes

Based on the substrate specificity, PPO enzyme is classified into three major classes. First class is called as catechol oxidase, diophenol oxidase or diphenol oxygen oxidoreductase (EC 1.10.3.1) which possesses both cresolase and catecholase activities [30, 47, 66]. Secondly, it is designated as laccase, or *p*-diphenol oxidase (EC 1.10.3.2) [30]. These enzymes possess only catecholase activity. They have the unique ability to catalyze the oxidation of *p*-diphenol or *o*-diphenol to form their corresponding quinones as shown in the figure 3.5. But the first class (EC 1.10.3.1) can catalyze only the oxidation of *o*-diphenols [66]. Laccases are less frequently encountered as a cause of browning in fruits and vegetables because they are almost absent in fruits and vegetables, except for peach and apricot [30, 66]. They mainly exist in fungi and in certain higher plants [66].

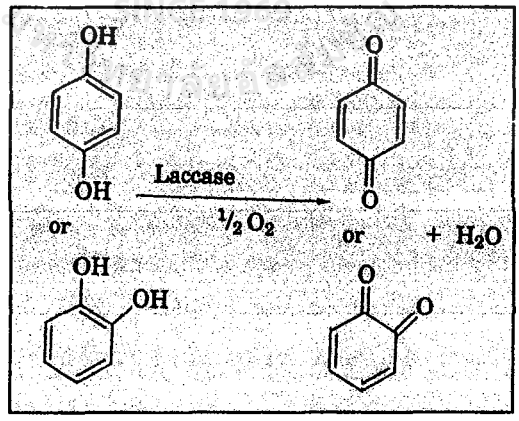

Figure 3.5: Laccase catalyzed reaction [30].

In addition, a third class is proposed as cresolase, tyrosinase, or monophenol monooxygenase (EC 1.14.18.1).These enzymes possess only cresolase activity with L-tyrosine as the major monophenolic substrate. However, they correspond to the same enzyme as EC 1.10.3.1. The first and third class are often referred by many authors to be PPO practically occurring in the plants.

3.2.2 Subsequent non-enzymatic reactions

At the end of the initializing step, *o*-quinones are derived as a final product. They possess a color which can be ranged as red to brown [99]. Differences in stability and color intensities of *o*-quinones are owing to the different phenolic substrate and environmental conditions [40, 47]. As *o*-quinones are highly reactive electrophilic molecules, *o*-quinones can participate in subsequent non-enzymatic reactions [53, 105]. They can further react with other quinones, with other phenolic compounds, with the amino groups of proteins, peptides and amino acids, with aromatic amine, and with thiol compound [31]. But the subsequent reaction most associated to brown discoloration in fruits is melanogenesis [47].

3.2.2.1 Melanogenesis

Melanogenesis is a process of biosynthesis of melanin in human skin and also in fruits. This reaction causes the discoloration of fruits. Quinones obtained from the enzymatic reaction catalyzed by PPO has involved on melanin synthesis by the formation of unstable hydroxyquinones. The hydroxyquinones readily polymerize and further being oxidized non-enzymatically to a dark brown pigment called melanin [30]. These melanins can further react with amino acids and proteins to enhance the intensity of brown color [58]. A typical melanogenesis occurs with tyrosine as substrate as shown in the figure 3.6. Tyrosine is hydroxylated into dihydroxylphenylalanine (DOPA) which is further oxidized into DOPA quinones. This step is catalyzed by PPO [30, 78]. DOPA quinones further undergoes a number of oxidations, and polymerization to form the various products including the melanin [105]. Melanins produced in the reaction can be classified into two kinds: pheomelanins (red to yellow) and eumelanin (dark brown) [105]. The brown melanin would contribute to the brown discoloration in the fruits.

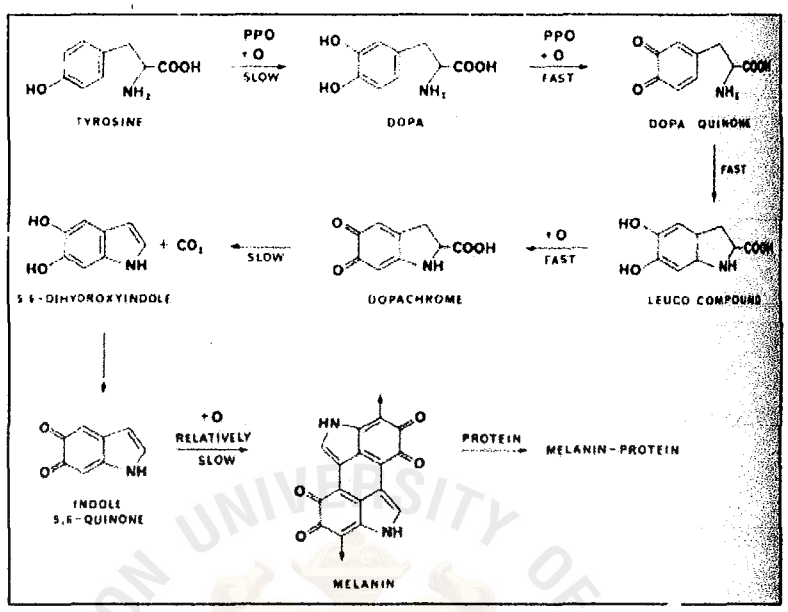

Figure 3.6: Melanin synthesis by tyrosine oxidation [105].

3.2.3 Major factors influencing the enzymatic browning

Regarding to the reaction, the rate and intensity of the enzymatic browning could be different from time to time depending on various internal and external factors. But the most influential factors are the activity of enzyme, concentration of specific phenolic substrates, oxygen availability, pH and temperature [68].

The major internal factors have included activity of PPO and phenolic contents in which higher extent of these factors lead to higher rate of the enzymatic browning [53]. On the other hand, the major external factors have included oxygen availability, pH and temperature. Oxygen is required in a sufficient amount to drive the reaction by acting as co-substrate. Oxygen is the first substrate to be bound by PPO enzyme. Regarding to pH and temperature, both are factors that have been focused on this research. Their influences on the enzymatic browning are described one by one as following.

pH

pH is one external factors affecting directly on PPO activity. The optimum of pH for PPO is reportedly ranged from acid to neutral (pH 5-7) whereas it is inactivated at pH below 4 [30]. But in apples, most studies indicate that apple PPO has optimum pH between 4.5 and 5.5 [66]. Moreover, this enzyme in apples seems to tolerate the acidic pH (pH 3) since the pH condition in apples is acidic [66]. However, PPO activities in apples would be dramatically retarded at pH below 3 and nearly ceased at pH 3.5 [11, 66].

Temperature

Temperature is another factor which mainly affects on PPO activity. Generally, the activity of PPO increases when the temperature rises from cold to optimum temperature which provides a maximum activity. Then the activity reduces when temperature goes beyond the optimum point. Temperature optimum and thermotolerance of PPO varies depending on the substrate specificity, pH, and also source of the enzyme [68]. Exposure of tissues to the temperature range from 70-90°C at short period is sufficient for partial or complete destruction of PPO. In apples, PPO optimally functions between 25 and 35°C and being inactivated at temperature higher than 40°C [66]. For example, a partially purified extract of apple PPO was reported to has half-life of 12 minutes at 65°C and be destroyed at 80°C [66]. Exposures to temperature lower than zero possibly affect the activity [30]. However, the enzyme slowly loses its activity in the frozen state [30]. In spite of PPO from the same source, they may have different thermotolerance due to the different molecular form of PPO. Laccase is usually more susceptible to heat inactivation than in catechol oxidase.

3.3 Inhibition of the enzymatic browning

The adverse effects of the enzymatic browning on a fruit quality have prompted a discovery of methods used to control the browning reaction. This becomes an important issue for minimizing loss and maintaining economical value in the fruit industry. One method, which is the most popular, is sulfurization.

3.3.1 Sulfurization

Enzymatic browning reaction can be retarded or inhibited by a process called "sulfurization" which is used to treat or impregnate a sample with sulfiting agents. Sulfites or sulfiting agents are widely used in the fruit industry because they are among the most effective agent for controlling brown discoloration in fruits [30, 72].

3.3.1.1 Forms of sulfiting agents

Sulfites are present in gaseous sulfur dioxide (SO_2) or several forms of inorganic sulfite salts which finally liberate SO_2 upon their use. The inorganic sulfite salts have included sodium and potassium metabisulfite ($Na_2S_2O_5$, $K_2S_2O_5$), sodium and potassium bisulfite ($NaHSO_3$, $KHSO_3$), and sodium and potassium sulfite (Na_2SO_3, K_2SO_3) [92]. Normally, the gas form can penetrate into fruits at faster rate than salt form but the sulfite solution prepared from salt form is more convenient to use for dipping or spraying in a processing plant [99].

As SO_2 from sulfite salts has dissolved in water, sulfurous acid (H_2SO_3) will form and exist as oxospecies including bisulfate (HSO_3^-) and sulfite (SO_3^{2-}) in aqueous solution that can be described by reaction equilibrium in the equation 3.1 [36, 58].

$$SO_2\,(g) + H_2O \leftrightarrow SO_2 \cdot H_2O\,(aq) \qquad (Eq.\ 3.1)$$

$$SO_2 \cdot H_2O \leftrightarrow [H_2SO_3]$$

$$[H_2SO_3] \leftrightarrow H^+ + HSO_3^-$$

$$HSO_3^- \leftrightarrow H^+ + SO_3^{2-}$$

The predominant ionic species depends on pH, ionic environment and water activity [58]. But bisulfate (HSO_3^-) mainly exists in the normal pH range in food and is maximized at acidic concentration around pH 4 [58, 106]. It is suggested that HSO_3^- is a main component for inhibiting PPO, which causes the browning [62].

3.3.3.2 Properties of sulfiting agents

Apart from the effective antibrowning activity based on antioxidant properties, multifunctional sulfites are able to act as the antimicrobial agent, and bleaching agent [72]. But the effectiveness of sulfite mainly contributes to the antibrowning and antimicrobial activity. Sulfites are highly used to prevent the microbial growth and spoilage in foods for long time [96]. The microbial inhibition performed by sulfites is owing to the disruption of alcoholic fermentation, reduction of disulfide linkage essential for enzyme activity, and reacting with cofactors, coenzymes, amino acids, pyrimidines and nucleotides [4, 106].

3.3.3.3 Mechanism on the enzymatic browning inhibition by sulfiting agents

The mechanism of sulfating agents to inhibit the enzymatic browning is still not completely understood now because of its complexity. However, it is hypothesized that the inhibition may be carried out by one of five possible following mechanisms which have been studied and proposed.

Firstly, sulfiting agents directly inhibit the action of polyphenol oxidase (PPO) in which bisulfite (HSO_3^-) possesses a competitive inhibitory effect on this enzyme [72]. *Garcia, et al. (2002)* and *Marshall, et al. (2000)* reported that HSO_3^- binds a sulfhydryl group at the active site of PPO and causes the irreversible structural modification preventing PPO to catalyze enzymatic browning reaction [31, 58]. For instance, *Chichester, et al. (1986)* reported that strawberry PPO is competitively inhibited by $K_2S_2O_5$ at 10 mM concentration [17].

Secondly, sulfites react with intermediates of the enzymatic browning reaction. They are able to form a complex molecule with diphenols and quinones thus these intermediates are prevented to participate in the further reactions that can lead to formation of the brown polymeric pigments [17, 72]. Nucleophilic addition of bisulfite to *o*-quinones leads to the formation of quinone-sulfite complexes called sulfonate as shown in the figure 3.7 [106].

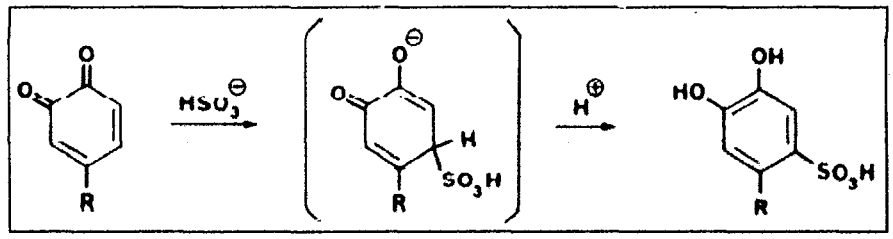

Figure 3.7: Formation of sulfonate from interaction between bisulfite and quinones [106].

Besides, *Martinez and Whitaker (1995)* has reported that reaction between sulfites and quinones can lead to the formation of sulphoquinones, which causes the inhibition of PPO [58].

Thirdly, sulfites are also capable to act as a reducing agent to reduce *o*-quinones back into less reactive and colorless diphenols [72]. Forth mechanism is based on the decreasing uptake of O_2 [47]. Sulfur dioxide (SO_2) causes the reduction of oxygen thus oxygen availability is not sufficient to drive the oxidative browning [26]. This oxygen reduction is suggested as a result of either direct oxidation of SO_2 or indirectly through quinones which oxidize SO_2 [26].

For the last mechanism, it is related to the ability of sulfites to neutralize free radicals produced during the enzymatic browning reaction. In the radical-mediated process of enzymatic browning, there is a formation of free radicals as shown in the equation 3.2 [11, 86].

Enzyme + Substrate ↔ Enzyme · Substrate (*Eq 3.2*)
Enzyme · Substrate + O_2 ↔ Enzyme · Substrate·O_2
Enzyme · Substrate·O_2 ↔ oxidized substrate + H^+ + Free radicals

Free radicals normally interact with another substrate to propagate the process as shown in the equation 3.3.

Free radicals + Substrate → Free enzyme + oxidized substrate + H_2O + OH^- (*Eq 3.3*)

But the concentration of these free radicals can be effectively decreased by bisulfite as shown in the equation 3.4 [86]. This is one possible reaction of sulfites to inhibit the enzymatic browning.

$$\cdot OH + HSO_3^- \leftrightarrow OH^- + HSO_3 \qquad (Eq\ 3.4)$$

Inhibition of enzymatic browning is a primary reason for using sulfites in various products such as shrimp, potatoes, mushrooms, apples, and other fruit and vegetable products [36]. The concentration of sulfites necessary for controlling the enzymatic browning varies in accordance with an activity level of PPO and substrate concentration in certain foods, time required for control of browning, and the presence of other inhibitors or controlling factors [92]. For example, a low level of sulfites are effective at the presence of monophenols while a higher concentration is required if diphenols are present [17, 92]. The concentration of sulfites is directly proportional to the length of time required for browning inhibition. Generally, a low level concentration of sulfite is sufficient enough to control the browning of fresh-cut apples in manufacture [17].

3.3.3.4 Advantages and disadvantages of sulfites

For advantages, sulfites possess multifunctional roles to preserve food quality. They are highly effective to perform their properties at relatively low concentration with the low cost of their application [62]. On the other hand, they also pose some limitations and drawbacks. Although they are effective on the enzymatic browning inhibition at trace amount, their concentration must be successively maintained if the long time of inhibition is required [58]. As sulfites are irreversibly oxidized by the reaction with quinones, the inhibition of enzymatic browning according to reducing properties of sulfites would be temporary [72]. For instance, once all sulfites are oxidized, the inhibitory effect is lost and then quinones will undergo further reactions to form brown pigments again [17]. Sulfites also partially deteriorate the sensory quality of foods by producing undesirable flavor, soft texture, and bleached color in some foods [B99]. Perhaps the most serious disadvantage of using sulfites in foods is due to their adverse effect on the destruction of vitamin B_1 (thiamine) so that they are banned in the foods rich in thiamine such as meat products [35, 99]. In the aspect of safety issue, FDA reported that the consumption of foods containing sulfites can promote acute allergic reaction in some of the asthmatic population [84]. In severe cases, it can cause the life-threatening anaphylactic-like reactions in some sensitive individuals [83]. Due to these undesirable effects, levels of sulfites are compulsorily reduced in many food products and they were already banned to use in fruits and vegetables by FDA [83, 92]. Finally, the search for potential sulfites alternatives is strongly necessitated.

3.3.2 Alternative substances or treatments for inhibition of enzymatic browning

The control of enzymatic browning is a challenging task for the food industry. Until now, considerable studies are extensively carried out to develop the strategies to replace the application of sulfur dioxide or sulfite salts in almost all food products [53]. The selection of methods depends on the type of product and intended use [30]. The basic step that can control the browning in fruits at the beginning is to choose cultivars which have a least browning susceptibility [53]. The food manufacturer would be responsible for this step. Besides, substantial physical and chemical methods have been employed for browning inhibition [47]. For physical methods, they are kind of processing techniques applied with fruits whereas chemical methods utilize the antibrowning activity of chemical compounds which can be categorized into reducing agents, acidulants, chelating agents, complexing agents, enzyme inhibitors, and enzyme treatments [37, 47]. The inhibition of the enzymatic browning by these alternatives generally occurs by the following three mechanisms. First is to directly inhibit the activity of PPO. Enzyme activity is inactivated by physical techniques or chemical compunds [72]. Chemicals either chelate the copper group of the enzyme, or act as the competitive inhibitors owing to their similar structure with phenolic substrates [58]. Secondly, the inhibition can be obtained from the removal of the substrates which are oxygen and phenolic compounds from the reaction [66]. But the complete removal of oxygen is most satisfactory method on the inhibition [66]. Normally, phenolic substrates are obstructed to involve in the browning reaction by complexation with chemicals or enzymatic modification [58]. Third mechanism is to act on the products especially *o*-quinones which can be either reduced back to diphenols or trapped as colorless compounds to prevent the formation of melanin [66, 72]. The processes and chemicals employed for controlling the enzymatic browning are grouped and shown in the table 3.1.

Table 3.1: Inhibitors and processes employed in the prevention of the enzymatic browning [37].

	Physical methods	**Chemical methods**
Inhibition targeted toward enzyme	Heating, Cooling, Dehydration, Irradiation, Ultrfiltration.	Chelating agents (azide, cyanide), Substituted resorcinols, Honey, Acidulants, Protease
Removal of oxygen	Vaccum treatment, Modified atmosphere packaging	Reducing agents (ascorbic and erythorbic acid, BHA, BHT), Edible coating
Removal of phenolic compounds	-	Complexing agents (cyclodextrin), Enzyme (*o*-methyl transferase)
Inhibition targeted toward products	-	Reducing agents (ascorbic acid, thiol compounds), Amino compounds, Chitosan

These methods can be applied solely or in combination to enhance the inhibitory effect. Many methods and chemicals are proved for their effectiveness for controlling the enzymatic browning. However, besides inhibitory effect, the preservation of the sensory quality and shelf-life is another important aspect for applying sulfite alternatives in fruit products [96]. Until now, there are no chemical agents or methods which can completely substitute the use of sulfite. Therefore, the investigation of potential alternatives is still needed. The ideal sulfite substitutes should be equivalent to sulfite in term of cost and effectiveness, meet safety standards and not contribute to undesirable sensory effects in products.

3.4 Plant extract

Because of the adverse effects of sulfite intake, inefficient sulfite substitutes, and risks of using synthetic compounds in food, there is an increment in the interest to use natural compounds for the enzymatic browning inhibition [91]. Therefore, the number of investigations on the natural antibrowning agents has been increased in the recent years. Plant derived substances are of interest due to their high availability, safety, multiple biological functions, and cost-effectiveness [100]. Plant extracts are potential to inhibit the enzymatic browning based on its secondary metabolites. Therefore, studies on antibrowning properties of plant extracts are more required.

3.4.1 Secondary plant metabolites

The beneficial effects of plant extracts are attributed to the properties function of secondary plant metabolites. They are bioactive compounds synthesized by plants for protection from herbivores and microbial infection, as the attractants to pollinators, as the alleopathic agents and as the UV protectants [19]. According to their biosynthetic origin, secondary plant metabolites can be classified into nitrogen and sulfur containing alkaloids, terpenoid compounds, and phenolic compounds [19]. But the phenolic compounds are the group which is supposed to perform antibrowning activity which can be mainly referred to their antioxidative properties [16].

3.4.2 Phenolic compounds

Phenolic compounds can be considered as phytochemicals which are ubiquitous in all parts of many plant varieties [38]. They are chemically characterized by an aromatic ring bearing one or more hydroxyl group and possibly containing functional derivatives such as esters, methyl ethers, glycosides, and so on [68]. Phenolic compounds can be divided into simple phenolics, phenolic acids, flavonoids, coumarins, stilbenes, tannins, lignans and lignins. The predominant phenolic compounds found in plant materials are gallic acid, quercetin, ellagic acid, tannic acid, ferulic acid, and anthocyanin [68]. Generally, these compounds are responsible for flavor and color of plants. Besides, they also provide the diverse beneficial biological activities including antioxidant and antimicrobial properties [1]. These properties contribute to the potential health benefits such as the reduction of fever and inflammation, relief of headache, and reduction of the risk for development of cardiovascular diseases and cancer [2, 55].

Until now, non-phenolic natural substances such as ascorbic acid, cysteine, and honey have been reported to be able to control the enzymatic browning based on antioxidant activity [58]. However, the application of these substances has some drawbacks such as the effect on sensory quality, temporary inhibition effect, etc. As phenolic compounds also have the antioxidant activity, they are possibly capable of inhibiting enzymatic browning [3].

3.4.3 Potential inhibitory effect of phenolic compounds on enzymatic browning

Based on the anti-oxidation, phenolic compounds could inhibit the enzymatic browning by the strong-hydrogen donation and free radical scavenging ability [68]. Regarding to these properties, phenolic compounds could inhibit enzymatic browning by acting on the substrates or products of the reaction. Phenolics could be able to exclude the oxygen from the reaction due to oxygen scavenging activity [58, 90]. They are able to scavenge reactive oxygen species by the electron and hydrogen donating properties [74]. As mentioned in 3.3.3.3, there is a formation of free radicals during enzymatic browning in which they can further bind with a new substrate to propagate the browning reaction again. Like sulfites, phenolic compounds could control enzymatic reaction based on the free radicals scavenging activity. They may stabilize the unpaired electron of the free radicals by donating a hydrogen atom usually from a phenolic hydroxyl group to the reactive radicals. Then, phenolic compounds would be converted into more stable and inactive free radicals. On the other hand, it could combine with other radicals to form inactive compounds that do not propagate further reaction as can be represented with the equation 3.5 [1, 64, 65, 90].

$$ROO\bullet + AH \longrightarrow ROOH + A\bullet$$
$$ROO\bullet + A\bullet \longrightarrow \text{Inactivated products}$$
$$A\bullet + A\bullet \longrightarrow \text{Inactivated products}$$

(AH was referred as Phenolic antioxidants) *(Eq3.5)*

The degree of antioxidant activity of phenolic compounds is influenced by the differences in the structure and substitution at hydroxyl group [104]. The phenolic compounds such as polyphenols which contain substantial hydroxyl groups, possess the strong antioxidant activity due to the high ability of hydrogen atom donation [57, 103]. On the other hand, the antioxidant activity decreases with an increase in glycosylation [103].

However, apart from antioxidant activity, phenolic compounds are proposed to inhibit the enzymatic browning by different other mechanisms which are mentioned in the following.

Phenolic compounds could directly inhibit the activity of PPO by binding at the active site of the enzyme [89]. For example, *Bourvellec, et al. (2004)* proposed that polyphenols such as procyanidins, which do not act as PPO substrate, could obstruct the catalytic activity of PPO [9]. These polyphenols would form the hydrogen bonds and hydrophobic interaction at the active site in which the larger polyphenols more strongly bind with proteins [9]. For this reason, the substrates are prevented to bind at the active site [9]. Phenolic compounds may inhibit the enzymes by the precipitation of enzyme proteins [34]. Besides, they might disrupt the enzyme activity by forming an inactive enzyme-polyphenol-substrate complex [9]. Phenolics could also be able to inactivate PPO via metal-chelation. Copper groups of enzyme can be complexed with phenolics through an unshared pair of electrons in their structure [24, 58]. *Soysal (2009)* reported that the green tea extract which contain substantial phenolic compounds exerts inhibitory effect on apple PPO by competitively binding at the active site due to structural similarity [91].

Due to the multiple potential mechanisms of phenolic substances on controlling the enzymatic browning, there is a possibility to use phenolic compounds or plant extracts which contain a considerable amount of these substances as the antibrowning agent [58]. Apart from phenolic content, the inhibitory effect of plant extract on the enzymatic browning is also influenced by the other substances in the extract, pH, product conditions, etc [58]. To illustrate the potential of using phenolics as the antibrowning agent, some phenolic compounds and their derivatives such as kojic acid, benzoic acid, cinnamic acid, substituted resorcinols, and phenolics in flavonones group have been found to inhibit the enzymatic browning effectively [58]. However, not all phenolic compounds could perform the inhibition on the enzymatic browning. On the one hand, some phenolic compounds act as PPO substrates and promote the enzymatic browning. Generally, common phenolic substrates for PPO are catechin, tyrosine, chlorogenic acid, cinnamic acid, catechol, pyrogallol, caffeic acid, *p*-hydroxycinnamic acid, *p*-cresol, and 3,4-dihydroxy phenylalanine (DOPA) [1, 106].

Therefore, the extensive studies on plant extracts containing phenolic compounds in the aspect of browning promotion as well as inhibition are necessitated in order to develop the potential sulfite substituts. As a crude extract of many herbs and spices are reported as a rich source of phenolic compounds, their extract could be used as one alternative of natural antibrowning agents. Clove

and lemon balm are two of many interesting herbs for investigation on the antibrowning properties since they contain a substantial amount of phenolic compounds [20, 104]. However, the phenolic composition in the extract is influenced by some extraction parameter such as type of solvent, the ratio between amount of solvent and plant material, and extraction temperature.

Clove

Clove (*Syzygium aromaticum*) is one of the most ancient and valuable spices belonging to the family of *Myrtaceae* [69]. The common synonyms are *Eugenia aromaticum* or *Eugenia caryophyllata* [71]. It is a tropical plant indigenous of Indonesia [71]. Normally, clove is practically used in the three forms: 1) whole or ground clove buds, 2) essential oils produced from bud, stem and leaf, and 3) clove oleoresins [71]. The major chemical compounds retained in all parts of clove are phenolic compounds especially eugenol and eugenol acetate (acetyl eugenol) which can be found in a considerable amount in clove oil [71]. Their structural configuration is illustrated in figure 3.8. The composition of clove oil comprises of approximately 71.56% eugenol and 8.99% eugenol acetate [15, 65]. Both are major compounds attributable to antioxidant activity [49].

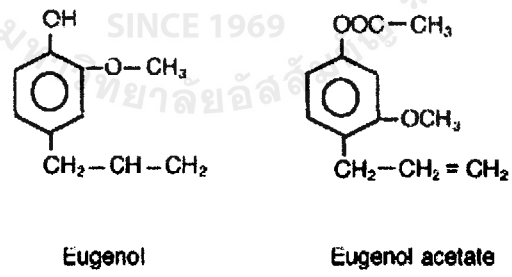

Figure 3.8: Chemical structures of eugenol and eugenol acetate [71].

Clove and its essential oil have the extremely high antioxidant activity in which its antioxidant capacity expressed as trolox is 346 µmol of trolox/100 g of dry weight [69, 104]. Antioxidant activity of clove oil at 0.005% is found to be equivalent to that of 0.01% BHT (common

antioxidants used in foods) [15]. Its high antioxidant activity enables clove to effectively scavenge free radicals especially hydroxyl radicals in which the activity is intensified with a higher concentration [43]. Inhibition of hydroxyl radicals by clove oil was found to be 91.2% at 0.6 μg/mL concentration. The antioxidant activity can mainly be attributed to a considerable amount of phenolics. Total phenolic content expressed as gallic acid equivalent (GAE) in clove is 14.38 g of GAE/100 g of dry weight [104]. Among these phenolics, eugenol and eugenol acetate are the main phenolics that provide the strong antioxidant activity due to their presence at substantial levels [49]. Eugenol could neutralize the hydroxyl radicals by donating a hydrogen atom form its phenolic hydroxyl group [43, 49]. But eugenol acetate does not have hydroxyl groups. Thus, it might performs the antioxidant activity based on the donation of a hydrogen atom from the methyl group (CH_3) linked to ester group to produce the stable radicals [49]. The antioxidant activity of eugenol and eugenol acetate is lower than that of tocopherol and BHT since they contain only one hydroxyl group [49, 104]. But as they are present at a substantial amount in clove, the antioxidant activity of clove extract could be comparable to those of known antioxidants [49]. Eugenol content in clove bud extract is reported as 9,382 mg per 100 g dry weight while eugenol acetate content is 2,075 mg per 100 g dry weight [104]. Apart from eugenol and its derivatives, the presence of phenolic acids like gallic acid, flavonols and tannins also significantly promote the antioxidant activity of clove extract [104]. They are recognized as the potent radical scavengers since they contain different kinds of hydroxyl groups especially ortho-dihydroxyl group [104]. In addition, they could directly inhibit PPO by complexation with copper at an active site [58, 84]. Moreover, benzoic acids like salicylic acid and others present in clove are known to be involved in the inhibition of the enzymatic browning [58, 70]. Besides phenolic compounds, the potential antibrowning activity of clove extract can be due to the presence of ascorbic acid in clove [71]. Clove contains ascorbic acid in concentrations of 80.81 mg/100 g which is recognized as a relatively high amount when comparing with the content of other vitamins in clove [71]. Ascorbic acid can inhibit the enzymatic browning by directly forming a complex with the prosthetic group of the enzyme, by reducing *o*-quinones to colorless diphenols, and by scavenging oxygen [2, 58, 72]. Alternative activity of this acid is to suppress the free radical formation which has involved in the browning reaction [2].

Lemon balm

Lemon balm (*Melissa officinalis*) is known as a medicinal plant belonging to the family of *Lamiaceae* that grows widely in the Mediterranean region [20]. However, it is cultivated all over the world owing to its culinary properties [20]. The part of lemon balm that is commonly used is leaves. The essential oils extracted from leaves are rich in phytochemicals contributing to various properties [64]. The major secondary plant metabolites present in lemon balm are phenolic compounds. It has a high level of total phenolics up to 13.2 mg of gallic acid equivalent (GAE)/100 g of dry weight in fresh lemon balm and 268.9 mg of GAE/g in lemon balm extract [14, 20, 104]. These phenolics are considerably present especially in leaves which contain phenolic acids up to 969 mg/100 g dry weight [104]. Among the main phenolics present in the leaves, hydroxycinnamic acids and their derivatives are counted for 11.29% of dry weight of leaf [64, 104]. Caffeic acid, protocatechuic and chlorogenic acid are the main hydroxycinnamic acids whereas the minor are *p*-coumaric and ferulic acid [20, 104]. But the most predominant phenolic compound is rosmarinic acid which is a derivative of caffeic acid. Its structure is illustrated in figure 3.9. The content of rosmarinic acid in lemon balm leaves is about 4.05% and in lemon balm extract (depending on the extraction method) is approximately 96.45 mg/g dry weight extract [14, 20]. Like eugenol in clove, rosmarinic acid is able to provide high antioxidant activity to inhibit enzymatic browning by donating a hydrogen atom to neutralize free radicals [20].

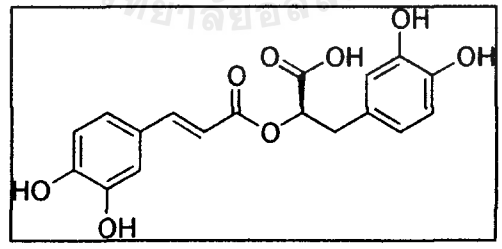

Figure 3.9: Chemical structure of rosmarinic acid [71]

Due to a high composition of rosmarinic acid, lemon balm exerts a high antioxidant activity in which its antioxidant capacity expressed as trolox is 10.6 µmol of trolox/100 g of dry weight [104]. The antioxidant activity of rosmarinic acid is reported to be greater than tocopherol and

comparable with BHA [14]. Lemon balm could perform this activity against enzyme-dependent and enzyme-independent oxidation [20]. As rosmarinic acid possesses several hydroxyl groups, lemon balm extract could be able to quench a wide range of synthetic and naturally occurring free radicals [64]. Apart from phenolic compounds, monoterpene aldehydes, ketones and squalene in lemon balm are found to show antioxidant activity [64]. Besdies antioxidant activity, the phenolic compounds in lemon balm could inhibit enzymatic browning by other means. Lemon balm might be involved in the inhibition of the enzymatic browning via the competitive inhibition and metal chelation [84]. The function of this mechanism is contributed by cinnamic acid and it derivatives, benzoic acid, condensed tannin and some phenolics in flavanols and flavanones groups present in lemon balm [14, 20, 109].

In conclusion, clove and lemon balm extract could serve as the potential natural antibrowning agent owing to various possible inhibition mechanisms mentioned above.

4. Material and Methodology

4.1 Materials

4.1.1 Equipment

4.1.1.1 Colorimeter

A Minolta colorimeter (Minolta Co. Ltd, Japan) is used to measure the color of the apple numerically to determine the degree of apple browning after treating with the extract. The following table (table 4.1) shows the specification of colorimeter used in this experiment.

Table 4.1: Descriptive specification of Chroma Meter CR-400

Name	**Chroma Meter Measuring Head**
Model	CR-400 Head
Detector	Silicone photo cells (6)
Display range	Y: 0.01 to 160% (reflectance)
Light source	Pulse xenon lamp
Measurement time	1 sec.
Minimum measurement interval	3 sec.
Measurement/illumination area	Ø 8 mm / Ø 11 mm
Illuminant	CIE C,D65
Size	102(W) x 217(H) x 63(D) mm
Weight	Approximately 570 g (Including 4 AAA size batteries and not including RS-232C cable)

4.1.1.2 Other instruments

The following table (table 4.2) is the list of all instruments used in the thesis project. The name of instruments is arranged alphabetically.

Table 4.2: Other instruments used in the research

Instrument	Specification
Autoclave	Adolf Wolf, SANO clave
Centrifuges	1. Heraeus, Biofuge Primo R 2. Beckman, J2-21 centrifuge
Freeze-dryer	Christ Alpha 2-4 LSC
Ice making machine	Scotsman AF-10
Laboratory balance	Mettler Toledo, Typ Classic
Magnetic stirrer	Janke & crony Ikawag RCT
pH meter	WTW, Microprocessor pH-Meter pH 537
Refrigerator	Liebherr, profi-line
Rotary evaporator	Büchi - Rotavapor R-114 - Water baht B-480
Water bath	Julabo PC

4.1.1.3 Standard laboratory equipments and household materials

The following tables (table 4.3 and 4.4) are the lists of all standard laboratory and household materials used in the thesis. The name of instruments is arranged alphabetically.

Table 4.3: Reusable standard laboratory and household materials

Equipment/Material	Specification
Apple cutter	-
Beakers	200 mL, 500 mL
Centrifuge tubes with caps	100 mL, 250 mL
Coffee making pot	Ikea
Cylinder	250 mL, 500 mL
Desiccator	-
Erlenmeyer flask	500 mL
Evaporator flask	2000 mL
Evaporator receiving flask	1000 mL
Magnetic bar	-
Magnetic rod	-
Porcelain mortar and pestle	-
Plastic basin	-
Plastic tray	-
Shott basin	-
Shott bottles with caps	500 mL

Equipment/Material (continued)	Specification
Spatula	-
Stirring glass rod	-
Stopwatch	VWR, count up/down timer
Thermometer	-
Water spray bottle	Made from PE, 500 mL

Table 4.4: Non-reusable standard laboratory and household materials

Equipment/Material	Specification
Aluminum foil	Commercial
Disposable petri dish	-
Disposable plastic pipette	Roth, disposable Pasteur pipettes, 1 mL, sterile
Foil cup	Ø 96 mm
Laboratory film	Parafilm
Emery paper	Lux
Zipper storage bag	Rewe

4.1.2 Raw materials

Apple

Apple was selected as a fruit used in the form of fresh cut to investigate the color change during enzymatic browning. "Jonagold" or "*Malus domestica*" is the apple cultivar used in this thesis. The apples were freshly brought from Obsthof Siebengebirge, Königswinter. The apples were purchased weekly. After one week, the old apples were disposed and the newly fresh apples were used instead of. They were stored in the closed room temperature cabinet which prevented the apple exposition to light when they were not used.

4.1.3 Chemicals

The following tables provide the name and brief specific information of all spice, plant extract and chemicals used in this investigation.

Table 4.5: Spice and plant extract

Spice/Plant extract	Used part of plant	Manufacturer	Solvent for extraction
Ground clove spice	Buds	Werner & Co.	-
Lemon balm extract	Whole plant	Frutarom	Water

Actually, the lemon balm extract was derived from the commercial manufacturer as mentioned in table 4.5 while the clove extract was prepared from the ground clove spice as will be explained further in the method part.

Table 4.6: Used chemicals

Chemicals	Manufacturer	Specification	Formula
Isopropanol	Roth	≥ 99.5%, Synthesis	C_3H_8O
Distilled water	IEL	Aquadem, Werner 0.1 $\mu S.cm^{-1}$	H_2O
Hydrochloric acid	Roth	37%, p.a.	HCl
Sodium hydroxide	Roth	≥ 99%, p.a.	NaOH
Sodium disulfite	Roth	≥ 97%, p.a.	$Na_2S_2O_5$

4.2 Methodologies

4.2.1 Extraction of antioxidant substances

For the inhibition of browning in fresh-cut apple in this thesis, plant extracts were investigated for an antibrowning activity. To perform this property, it is mainly due to the phenolic substances in the plant or spice in which it provides antioxidant activity to retard browning reaction. Therefore, the phenolic substances must be extracted from the plant, spice or herb before using as an extract. Ground clove spice derived from manufacturer must undergo extraction process but the lemon balm was already extracted by a commercial manufacturer.

4.2.1.1 Extraction of antioxidant substances from ground clove spices

Extraction

Twenty gram of fine ground clove spice was weighed with laboratory balance and added into a Erlenmeyer flask. The solvent was prepared as a mixture between isopropanol and distilled water at the ratio 1:1. Total volume of mixed solvent was 200 mL. Therefore, there was 100 mL for each solvent. Then, 100 mL of distilled water and 100 mL of isopropanol were added respectively into the Erlenmeyer flask which already contained the ground clove spices. The solution was stirred vigorously by a stirring glass rod until the solution was homogenous. The top of the Erlenmeyer flask was closed with aluminum foil for avoiding evaporation of solvent during the extraction. At each time of extraction, three flasks of solution were prepared.

The water in a water bath was heated up into 50 °C observed with thermometer. The solution containing Erlenmeyer flasks (three flasks) were put in the water bath and heated for one hour. During heating, the solution in each the Erlenmeyer flask was stirred with the stirring glass rod at every 15 minutes interval until reaching one hour. After one hour, the Erlenmeyer flasks were taken out from the water bath. After extraction, the solution from Erlenmeyer flask was added to centrifuge tubes but leaving the residue clove spice powder to settle down at the bottom of Erlenmeyer flask. The solution was transferred until reaching volume as 45 mL and then the lid was closed tightly. The solution was centrifuged for 10 minutes at 8000 rounds per minute (rpm). After centrifugation, the supernatant was collected and put in shott bottles while the pellet was disposed. Afterwards, the supernatant solution was evaporated in a rotary evaporator. But if the centrifugation and the evaporation were performed in different day, the supernatant solution would be stored in the fridge at the day of centrifugation for further evaporation process.

Removal of isopropanol by evaporation

The evaporation was done in a rotary evaporator (or rotavap) to remove the isopropanol retained in the solution. A 300 mL of centrifuged extract solution was measured and added into the an evaporator flask. Water in the water bath was heated up to 45°C. The vacuum condition was then activated. The sample was evaporated and spin simultaneously at 45°C for one hour. During evaporation, isopropanol was evaporated under reduced pressure and condensed into a receiving

flask by a condenser coil containing cool water inside. At the end of evaporation, the solution would contain less than 5% of isopropanol. The solution was kept in shott bottles and stored in a fridge before freeze drying.

Drying of extract by lyophilization

After evaporation, the solution from shott bottles was transferred to plastic petri dishes in which the solution was added to 1/3 height of each petri dish and the cover was closed. The petri dishes were kept in the freezer at -70°C for 1 day. Then, the petri dishes were put in a freeze dryer. The sample was lyophilised in the freeze dryer at - 94 °C for 4 days.

After freeze drying, the petri dishes were put in a desiccator to prevent the moisture absorption. Three petri dishes were taken out at a time from the desiccator. The dried clove extract was immediately taken out and ground into fine particles. The fine ground clove extract was then immediately put in a container and properly packed. Finally, the packed extract was kept in the zipper storage bag which would be labeled with name and date and stored in the freezer at -70°C until using.

4.2.2 Preparation of extract and sulfite solution

In this part, clove and lemon balm extract, and sulfite solution were prepared. Both of extract solutions must be prepared at three varied concentration (0.1, 0.55 and 1% (w/v)) whereas sulfite solution is prepared at 0.14% by using sodium bisulfite with distilled water as a solvent. Since at least approximately 250 mL of the extract and sulfite solution was required for the apple slices immersion, the volume of solvent would be 300 mL. The formula for each concentration of extract and sulfite solution is shown in the table 4.7.

Table 4.7: Formula of extract and sulfite solution

Extract solution concentration (% (w/v))	**Amount of powdered extract (g)**	**Volume of distilled water (mL)**
0.1%	0.3	300
0.55%	1.65	
1%	3	
Sulfite solution concentration (% (w/v))	**Amount of sodium bisulfite (g)**	**Volume of distilled water (mL)**
0.14%	0.42	300

To prepare the extract solution, the powdered extract of clove or lemon balm was weighed according to the required solution concentration and added into shott bottles. Then, distilled water was measured for 300 mL and added into the shott bottles which already contained the powdered extract. The extract solution was shaken vigorously by manpower until the dissolution of powdered extract was complete.

The extract solution was sterilized in an autoclave at 121°C for 1 hour to avoid a microbial contamination. Then, the extract solution was cooled down. After cooling down, the extract solution was transferred into centrifuge tubes. The lid was closed tightly and the solution was centrifuged at 8000 rpm for 10 minutes. The supernatant was collected into the shott bottles in which each bottle contained 300 mL of the extract solution. After this step, it can be stored in fridge for further pH adjustment but it must not exceed one week otherwise it must be stored in a freezer.

The next step was pH adjustment. Before adjusting pH, the temperature of the extract solution had to be adjusted to room temperature or about 21°C. pH of extract solution was varied into 3, 6.5 and 10. For pH 3 and 6.5, the solution's pH was adjusted with 0.1 N HCL solution, for pH 10, the solution's pH was adjusted with 1 NaOH solution. Adding HCL and NaOH solution was done drop by drop with plastic pipette. pH and temperature of extract solution after adjustment were recorded. The solution were stored in a fridge before using to treat with apple slices.

To prepare sulfite solution, the powder of sodium bisulfite is used instead of the extracts. Steps for preparation of sulfite solution are as same as the extract solution except that the sulfite

solution did not undergo the centrifugation and pH adjustment. Therefore, after cooling down from the sterilization, the sulfite solution was kept in a fridge before use.

4.2.3 Design of experiments

In this thesis, Design of experiments (DOE) was used to construct the all possible experiments for verifying the effect of studied factors on the inhibition of browning reaction. It was also used as a statistical tool to analyze, evaluate and optimize the results.

DOE is an approach planned to use for determining the relationships between cause and effect in which it is normally used to determine and study the effect of factors on responses in a process. It is one kind of applied statistics widely used in many fields and processes such as quality control, assurance and improvement for agricultural purposes, industries process, product development, etc [5, 21].

Two-level factorial design is used as a primary tool for DOE. It is a design in which each factor contains two levels that can be described as high and low level. Two-level full factorial design was used in this thesis in which every level of every factor were fully combined to each other so that all main effects and interactive effects can be analyzed precisely [5, 21]. Normally, the factorial design is used for many purposes such as screening through many factors to find out the critical few, estimation of all main effects and all interactive effects which cannot be obtained from one-factor-at-a-time (OFAT) method. It helps to gather the information as much as possible with the minimum of experiment runs. If three factors are involved in the study, the total runs from OFAT will be 16 which is much higher than in two-level factorial design that requires only 8 runs of experiment with the equivalent power [5, 21].

4.2.3.1 Construction of Two-level factorial design

Two-level full Factorial Design for three independent factors was adopted. To study the effect of clove and lemon balm extract on the inhibition of browning reaction in fresh-cut apple, there were three major factors involved: extract solution concentration, temperature, and pH. In each factor, it was varied into two levels as shown in the table 4.8. For the responses, since color

change is one effect occurring from browning reaction, total color difference (ΔE^*) and relative total color difference ($\Delta(\Delta E^*)$) were used as responses.

Table 4.8: Plan summary of Two-level factorial design

Two-level factorial design							
Factor	Name	Type	Levels		Midpoints	Response	
			Low Actual	High Actual		Y_1	Y_2
A	Extract concentration	Numeric	0.1%	1%	0.55	ΔE^*	$\Delta(\Delta E^*)$
B	Temperature	Numeric	10°C	40°C	25		
C	pH	Numeric	3	10	6.5		

The selection of low and high levels for all variables can be explained as following. For all variables, the levels were selected based on the previous works and screening test done in our laboratory.

For concentration, it was found in the previous work that the color of treated apple slices at concentration greater than 1% was obviously too dark and not suitable for consumption. Since the other works on the inhibition of browning reaction in fresh-cut apples used the minimum concentration of chemicals or extract solution not lower than 0.1%, the minimum concentration of extract solution in this experiment would be also 0.1%. For pH, since the pH optimum of apple polyphenol oxidase is between 4.5 and 5.5 [91], the pH level at 3 and 10 was selected in order to inactivate this enzyme in synergism with the extracts. Like pH, the level of temperature was also selected for the polyphenol oxidase inactivation. Normally, polyphenol oxidase has optimum temperature about 25 – 35°C and it is not heat stable enzyme so the temperature at 10 and 40 °C might improve the inactivation of this enzyme to prevent the browning reaction [66]. The treatments with extracts were compared in term of effectiveness on inhibiting enzymatic browning reaction in fresh-cut apples with sulfite solution treatment. The treatment with sulfite solution was performed at 0.14% concentration, and room temperature.

According to the design of experiments shown in table 5.8 the total number of runs from two-level full factorial design with three factors was 2^3. Since two replicates were done at the center point, the total number of experiments was 10 as shown in the table 4.9.

Table 4.9: Two-level full factorial design

Standard	Run	Factor A Concentration	Factor B Temperature	Factor C pH
3	1	0.10	40.00	3.00
6	2	1.00	10.00	10.00
1	3	0.10	10.00	3.00
10	4	0.55	25.00	6.50
9	5	0.55	25.00	6.50
8	6	1.00	40.00	10.00
5	7	0.10	10.00	10.00
4	8	1.00	40.00	3.00
7	9	0.10	40.00	10.00
2	10	1.00	10.00	3.00

4.2.4 Color measurement

4.2.4.1 L*a*b* color system

Since the color change is one consequence occurring from the browning reaction, color measurement based on L*a*b* system can be used to detect such change in order to determine the degree of browning. For color measurement, it was done with colorimeter.

Color is one aspect of appearance based on visual perceptual property that response to light [107]. Color composes of three attributes: hue (color), lightness (brightness) and saturation (vividness) [45]. Hue is a term used to classify the color into red, blue, green, or mixing of color such as orange (yellow-red), violet (red-blue), blue-green, etc [45]. Lightness is the luminous intensity of color used to describe the degree of darkness and brightness of color. For example, the yellow of lemon is brighter than the red of cherry [45]. Saturation is a term used to describe the vividness or dullness of the color. For example, when comparing the saturation of red color

between tomato and radish, the red of tomato is more saturated or vivid than the red of radish [45].

To perceive the color, it requires three components: light, vision and object. Normally, when human see the color, it is originated from the interaction of these three components. Light radiation is hitting on an object in which some of light radiation is absorbed by pigments in the object while the remaining is reflected back to the observer's eyes. This reflected light stimulates the color receptor cells in the retina in eyes in which the human color receptor cells are sensitive to a visible spectrum at 400-700 nm. Then, the information is sent to brain for interpretation as color [45]. This is the reason that human can identify and differentiate the colors. However, people can see, interpret and define the color of the same object as different because of the differences in light source, background, direction of observation, size of object, personal references and sensitivity of observer's eyes [45]. Thus, this can lead to many color-related problems. To eliminate such problems, the accurate color communication practice was developed to identify, differentiate and express color as numerical value. For this reason, a term of color space was originated. Color space is a method for quantifying and expressing the color of an object or a light source numerically in order that color communication can be performed easily and accurately. At the present, many color spaces have been devised and one of the most well known and widely used is L*a*b* color system. L*a*b* color system (CIELAB) was devised and developed from Yxy color space by CIE (Commission internationale de l'éclairage) in 1976 with the aim to provide a uniform color scale in which the equal differences between points plotted in color space correspond to the perceived color differences [39]. It is organized to three dimensional diagram consisting of three coordinates, which are L*, a* and b* coordinate. Coordinate L* indicates lightness while coordinates a* and b* indicates the chromaticity.

For a* and b* coordinates, they do not have the numerical limit. They have only positive and negative with varying extent. For a* coordinate, it defines redness and greenness in which +a* is the red direction and –a* is the green direction. For b* coordinate, it defines yellowness and blueness in which +b* is the yellow direction and –b* is the blue direction [39]. The intersection between a* and b*in color space identifies the chromaticity in term of hue and saturation as can be seen at point A and B in figure 4.1 [45]. For both a* and b* coordinates, when a* and b*

value increases either in positive or negative direction, the saturation of color increases [45]. As can be seen in figure 4.1, the color is more saturated when the point moves out from the center indicating higher a* and b* value.

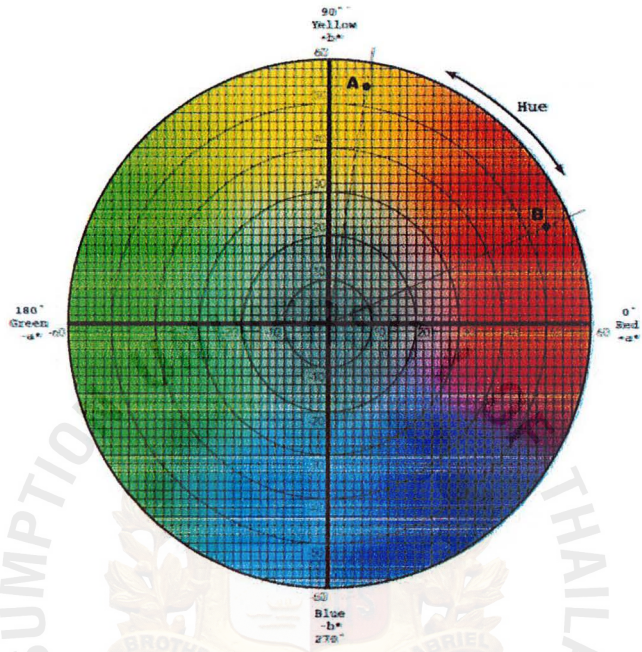

Figure 4.1 : 2-dimensional (a* and b* coordinates) CIELAB color chart.

But when coordinate L* is added, the complete color space is obtained as shown in the figure 4.2. For coordinate L*, the lightness coordinate runs from the top to bottom. The maximum value for L* is 100 locating at the top of the coordinate that represents white or total reflection while the minimum is 0 locating at the bottom of the coordinate that represents black or total absorption [45]. At the center of the color space or the intersection of three coordinates, it is achromatic or neutral [107]. The diagram representing CIELAB color space is shown in the figure 4.2.

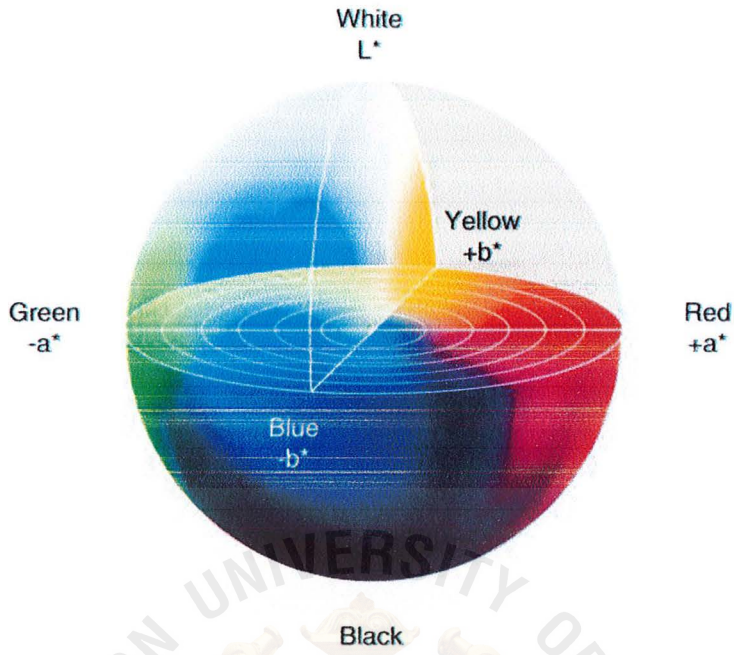

Figure 4.2: CIELAB color space

The intersection of these three values gives the specified color of an object so it can be used to measure the color of an object including apples which are the subject of this experiment.

Since human cannot quantify colors accurately into numerical value, the colorimeter was developed. Colorimeter is an optical instrument used to measure color by filtering the reflected light into red, blue and green dominant regions. It uses tristimulus method to measure the color of an object which is the same with how human perceives the color because the sensors of colorimeter have the same color sensitivity as human eyes. Since it always uses the same light source and illumination method for every color measurement, it can express color into a precise numerical value with the principle of color space behind that. It can also detect slight difference in color. Besides, it also has many comparative advantages such as low price, compact size, superior mobility and simple operation [45].

Apart from the color identification, the indication on how much a sample's color differs from the standard or reference color is also important and widely used especially in the quality control and formula adjustment. Therefore, the term "color difference" was developed. Since the color of

apples must be compared with the reference for four hours to determine the degree of browning, a color difference is important and must be identified.

The color difference can be expressed as ΔL^*, Δa^* and Δb^* which is the difference in L*, a* and b* value respectively between the sample and reference. To calculate each term of color difference, the formula are shown as following:

$$\Delta L^* = L^*_{sample} - L^*_{reference}$$
$$\Delta a^* = a^*_{sample} - a^*_{reference}$$
$$\Delta b^* = b^*_{sample} - b^*_{reference}$$

For the interpretation of each delta value, if ΔL^* is positive, it means that a sample is lighter than reference vice versa. For Δa^*, positive value of Δa^* indicates that a sample is redder than the reference while negative value of Δa^* indicates that the sample is greener than the reference. For Δb^*, positive value of Δb^* indicates that a sample is yellower than the reference while negative value of Δb^* indicates that the sample is bluer than the reference.

However, the total color difference (ΔE^*) was used in this experiment to determine the degree of browning. ΔE^* is a single numerical value indicating the differences in L*, a* and b* value of color between a sample and reference. It can be calculated by using the following formula:

$$\Delta E^* = \sqrt{\Delta L^{*2} + \Delta a^{*2} + \Delta b^{*2}}$$

This value indicates only the magnitude of color difference but not in what way of L*, a* or b* is different from the reference.

In the experiment, the browning reaction was observed and determined for four hours with comparison with the reference which is the apple slices' color immediately measured after 10 minutes dipping. When apple slices were exposed to atmosphere, it naturally underwent the enzymatic reaction resulting in the color of apples slice became browner than the reference. Consequently, L* and b* value decrease while a* value increases so that $\Delta L^*$and Δb^* is more negative while Δa^* is more positive with an increasing degree of enzymatic browning. The

changes of these three parameters totally contribute to a higher ΔE* value [52]. Therefore, the higher ΔE* can imply the higher degree of browning. Different range of ΔE* value has the difference implication as shown in table 4.10:

Table 4.10: Delta E* range scales and their implication [52]

Scale	Implication
ΔE* < 0.2	Not perceptible difference
0.2 < ΔE* < 0.5	Very small difference
0.5 < ΔE* < 2	Small difference
2 < ΔE* < 3	Fairly perceptible difference
3 < ΔE* < 6	Perceptible difference
6 < ΔE* < 12	Strong difference
ΔE* > 12	Different color

Apart from ΔE*, The relative total color difference (Δ(ΔE*)) was conducted to determine the difference of total color difference (ΔE*) between the apple slices treat with extract and water control. Therefore, it can be used to compare and assess the efficiency of the extracts and water on inhibiting the browning. Δ(ΔE*) can be calculated by using the following formula:

$\Delta(\Delta E^*) = \Delta E_{control} - \Delta E_{extract}$

4.2.4.2 Antibrowning tests with fresh cut apples

For the color measurements, the apple slices were dipped into extraction solutions, namely clove and lemon balm extract solution. Then, the degree of browning was investigated by using a colorimeter to measure the color of the apple flesh that color was expressed as L*a*b* value. Before each measurement, the colorimeter was calibrated with the white calibration plate at at L * = 80.02, a * = -4.48 b * = 24.53

Treatment of apple slices

Firstly, A 300 mL of the extract solution was put into the coffee making pot. The solution's temperature was adjusted according to the design of experiment to 10, 25 or 40 °C. To raise the temperature of extract solution, a water bath was applied while the reduction of solution temperature was facilitated with ice water. The temperature of extract solution was adjusted until reaching the desired temperature. Then, an apple was cut by an apple cutter into eight equally thick slices. Five of eight slices were roughened with emery paper to ensure the uniform browning on the surface of apple flesh. Upon roughing, the tissue and cells of apple flesh was damaged and open so that it allowed polyphenol oxidase to come out and react with substrates. Three from five roughened apple slices were immediately placed and submerged in the extract solution stored in a coffee making pot while the other 2 roughened apple slices were submerged in a tap water bath. At this point, a time recording was started. A dipping time was 10 minutes. While waiting for dipping, one of un-roughened apple slices was taken to measure the initial L*a*b* value of natural apples. For the measurement, the measuring head of colorimeter was placed perpendicular to apple flesh and at the center of apple slices. The measurement was taken as triplicate and the results were calculated as an average.

Apart form the treatments with extracts, apple slices are also treated with 0.14% sulfite solution according to the conformance with *Gemüse Meyer* who is the industrial fresh-cut fruit manufacturer and our project partner. Since the application with sulfite solution is a regular treatment industrially practiced by manufacturer, the effect from pH and temperature, and the optimization of this application is not necessary to be considered. Therefore, the treatment with sulfite solution can be carried out at room temperature without heating or cooling.

Color measurement of apple slices during exposure to air

When reaching 10 minutes of dipping, three apple slices were taken out from the extract or sulfite solution, and one apple slice was taken out from water. All four apple slices were dried and placed on a tray in which the first position was the apple dipped with tap water that it was used as a control. Then, the color measurement on each apple slice was performed immediately by using the colorimeter. This measurement was recognized as the first time of measurement,

and L*a*b* values of this first measurement was used as a reference value used to calculate ΔE* value. To complete the treatment, apple slices were exposed to air at room temperature and measured for 4 hours. After the first color measurement, color of apples slices was measured at every 5 minutes interval until reaching first one hour. At the second hour, they were measured at every 10 minutes interval. At the third and forth hour, they were measured at every 20 minutes interval. The L*a*b* values of each measurement were recorded in a data sheet for ΔE* calculation.

After complete the measurement, ΔE* values for samples and control were calculated to asses the degree of browning in which the greater ΔE* value implies the greater extent of browning reaction. The highest ΔE* value of samples and control was picked up from the last hour of measurement. To compare the degree of browning between samples and control, Δ(ΔE*) value was used and calculated. Δ(ΔE*) value is defined as the difference between the ΔE* value of the control and ΔE* value of sample treated with the extract ($\Delta(\Delta E^*) = \Delta E_{control} - \Delta E_{extract}$). The greater Δ(ΔE*) value indicates the better efficiency of the extract on inhibiting the browning reaction when comparing with water control.

4.2.5 Statistical analysis

All experiment data derived from the full factorial design were analyzed by using Analysis of Variance (ANOVA). ANOVA is a method of statistical analysis of quantitative data. The analysis is made based on the variance which is a primary statistic used to measure the variability of data distribution. This variance is partitioned into the corresponding components, and compared to find out whether the difference of experiment data is due to the effect of factors or background noise (natural variation). The probability of obtaining significant difference derived from factor effects or natural variation is referred as p-value. P-value at $P < 0.05$, $P < 0.01$, and $P < 0.001$ means that one or more of the factors or treatments have contributed a significant effect on the measured response with 95%, 99% and 99.9% confidence respectively. In other words, it means that there are 5%, 1% and 0.1% chance respectively that the variation or the difference among the data is due to the natural variation. All analysis in this work were made at $P < 0.05$.

As a result of analysis, the statistical model was shown for explaining the variation and predicting the response. The effectiveness for using the model on the prediction can be judged by

the coefficient determination (R^2) ranging from 0 to 1. The ideal model, which can give the predicted value completely correlated with measured value, is assigned with R^2 at 1. In addition, the corrected coefficient of determination (Adjusted R^2) and the predicted coefficient of determination (Predicted R^2) are determined and they should be coinciding with R^2.

Lack of fit is another parameter used to determine the reliability of the model. Lack of Fit is considered as the variation of the data around the fitted model. If the lack of fit is significant, it implies that the model does not fit with the data well. In the other words, it may have another model which is better to fit for use.

4.2.6 Optimization

After performing two-level factorial design to quantify the correlation between the factors and responses, the optimization was taken into an action to find the most suitable combination of three factors for providing the best effect on the enzymatic browning inhibition.

Optimization is a technique used to find the combinations of a number of experimental factors that will lead to an optimum response which can be maximum or minimum depending on its nature. As responses in this experimental design were ΔE^* and $\Delta(\Delta E^*)$, ΔE^* should be minimized while $\Delta(\Delta E^*)$ should be maximized for optimization. The minimum ΔE^* and maximum $\Delta(\Delta E^*)$ indicate the greatest efficiency on inhibiting browning reaction. [5, 21].

For optimizing the responses, one response optimization and simultaneous optimization of two responses were performed. The optimization for one response is defined as "univariate optimization" while the simultaneous optimization for more than one responses is defined as "multivariate optimization". For one response optimization, the highest importance was given for ΔE^* (number 1 in table 4.11) or $\Delta(\Delta E^*)$ (number 2 in table 4.11). For two responses optimization, the importance was given for both ΔE^* and $\Delta(\Delta E^*)$ equally (number 3 in table 4.11). The optimized designs were selected for both clove and lemon balm extract with a criteria of the highest desirability and most satisfying the desired specifications. There are three optimized designs for each plant extract as shown in table 4.11.

Table 4.11: Designs for one response optimization (ΔE* or Δ(ΔE*)), and simultaneous two responses optimization (ΔE* and Δ(ΔE*)).

Colve Optimization						
Number	**Concentration**	**Temperature**	**pH**	**Predicted ΔE***	**Predicted Δ(ΔE*)**	**Desirability**
1	1.00	39.17	3.01	1.48619	-	1
2	1.00	10.00	3.01	-	3.87751	0.69434528
3	1.00	36.53	3.00	1.5547	3.51899	0.78578822
Lemon Balm Optimization						
Number	**Concentration**	**Temp.**	**pH**	**Predicted ΔE***	**Predicted Δ(ΔE*)**	**Desirability**
1	0.10	39.65	9.93	3.84801	-	1
2	0.10	38.47	5.08	-	1.61371	0.91831382
3	0.10	39.62	9.96	3.8171	1.61370	0.95828655

5. Results

5.1 Full factorial experimental plan

According to the full factorial design in the part of method (section 4.2.3), the plans were performed in a number of experimental runs to obtain the value of response variables: the total color difference (ΔE*) and relative total color difference (Δ(ΔE*)). The results of responses were input in the experimental design of the software Design-Expert ® version 6.0.5 for statistical purposes.

5.1.1 Full factorial results

The results of the ΔE* and Δ(ΔE*) parameters with the corresponding two-level factorial plan for lemon balm extract are shown in the table 5.1.

Table 5.1: The results of ΔE* and Δ(ΔE*) values of apple slices treated with lemon balm extract.

Lemon balm extract		Factor A	Factor B	Factor C	Response 1	Response 2
Std	Run	Extract Concentration (%)	Temperature (°C)	pH	ΔE*	Δ(ΔE*)
3	1	0.10	40.00	3.00	6.71	2.35
6	2	1.00	10.00	10.00	10.64	-6.31
1	3	0.10	10.00	3.00	10.00	0.85
10	4	0.55	25.00	6.50	15.26	-6.01
9	5	0.55	25.00	6.50	15.71	-5.68
8	6	1.00	40.00	10.00	8.36	-5.27
5	7	0.10	10.00	10.00	6.20	1.94
4	8	1.00	40.00	3.00	10.60	-5.22
7	9	0.10	40.00	10.00	3.95	1.32
2	10	1.00	10.00	3.00	14.13	-6.66

The results of the ΔE* and Δ(ΔE*) parameters with the corresponding two-level factorial plan for clove extract are shown in the table 5.2.

Table 5.2: The results of ΔE* and Δ(ΔE*) values of apple slices treated with clove extract.

Clove extract		Factor A	Factor B	Factor C	Response 1	Response 2
Standard	**Run**	**Extract Concentration**	**Temperature**	**pH**	**ΔE***	**Δ(ΔE*)**
3	1	0.10	40.00	3.00	2.76	2.19
6	2	1.00	10.00	10.00	3.60	1.88
1	3	0.10	10.00	3.00	5.02	2.33
10	4	0.55	25.00	6.50	2.21	5.30
9	5	0.55	25.00	6.50	2.54	5.25
8	6	1.00	40.00	10.00	4.89	2.83
5	7	0.10	10.00	10.00	5.43	0.64
4	8	1.00	40.00	3.00	1.55	3.29
7	9	0.10	40.00	10.00	5.97	0.96
2	10	1.00	10.00	3.00	2.72	3.96

5.1.2 ANOVA analysis of the results

After the results were obtained from the factorial plans, the results of both ΔE* and Δ(ΔE*) were then analyzed by ANOVA with the help of statistical software. ANOVA was performed to figure out the significant factors which give the impact on both ΔE* and Δ(ΔE*) through main and interactive effect. The best suited model of significant factors was constructed by ANOVA. In this experiment, the linear model was chosen by the software with p-value < 0.05 for both ΔE* and Δ(ΔE*) of apples treated with clove and lemon balm extract. The p-value less than 0.05 implied that there is more than 95% confidence that the responses are significantly affected by one or more of the factors chosen for the model. The results of ANOVA for ΔE* and Δ(ΔE*) from both extract are presented in the tables 5.3 to 5.6.

Lemon balm extract

The results of ANOVA for ΔE* from the lemon balm extract are shown in the table 5.3.

Table 5.3: ANOVA for the response ΔE* of the apple slices treated with the lemon balm extract.

Source	F-Value	Prob>F	R^2	Adjusted-R^2	Predicted-R^2
Linear Model	136.08	< 0.0001	0.9879	0.9806	0.9757
A	205.91	< 0.0001			
B	93.09	0.0002			
C	109.25	0.0001			
Lack of fit	1.88	0.4941			

ANOVA results in table 5.3 showed that the model fit for response ΔE* was the linear model. The model was significant with the main effect of lemon balm extract concentration (A), temperature (B) and pH (C) at p-value < 0.0001 while there was no significant interactive effect between any factors. Analyzing individual effect, each main effect was also significant as well at p-value < 0.05. The lack of fit of this model was not significant with F=1.88 and P = 0.4941. The non-significance of lack of fit indicated the fitness for use of the model for predicting the response outcomes. The coeffiecient of determination (R^2) in this model was 0.9879 which coincided with adjusted- R^2 at 0.9806 and predicted- R^2 at 0.9757.

The coeffiecient of determination (R^2) is the total variation that can be explained by the statiscal model. As R^2 indicates the correlation between the measured responses and predicted responses, it can also imply the ability of the model for prediction of the response. As R^2 in this model was 0.9879, it means that the 98.79% of the variation of ΔE* response can be explained or predicted by this model.

The results of ANOVA for Δ(ΔE*) from the lemon balm extract are shown in the table 5.4.

Table 5.4: ANOVA for the response Δ(ΔE) of the apple slices treated with the lemon balm extract.*

Source	F-Value	Prob>F	R^2	Adjusted-R^2	Predicted-R^2
Linear Model	262.94	< 0.0001	0.9741	0.9704	0.9605
A	262.94	< 0.0001			
Lack of fit	8.81	0.2524			

ANOVA results in table 5.4 showed that the linear model fitted for response Δ(ΔE*) which was significantly affected by only main effect of lemon balm extract concentration (A) at p-value < 0.0001. The lack of fit was not significant with F=8.81and P = 0.2524. The coeffiecient of determination (R^2) in this model was 0.9741 which coincided with adjusted- R^2 at 0.9704 and predicted- R^2 at 0.9605.

Clove extract

The results of ANOVA for ΔE* from the clove extract are shown in the table 5.5.

Table 5.5: ANOVA for the response ΔE* of the apple slices treated with the clove extract.

Source	F-Value	Prob>F	R^2	Adjusted-R^2	Predicted-R^2
Linear Model	30.58	0.0029	0.9683	0.9367	0.8269
A	37.92	0.0035			
B	2.33	0.2014			
C	56.42	0.0017			
BC	25.63	0.0072			
Lack of fit	3.05	0.3929			

ANOVA results in table 5.5 showed that the model was significant with the linear model. The main effect of the clove extract concentration (A) and pH (C), and interactive effect between temperature and pH (BC) were significant on ΔE* at p-value < 0.05. Although the main effect of

temperature (B) was not significant with p-value > 0.05, this effect was included in the model for the compatibility of hierarchy. For this model, the lack of fit was not significant with F=3.05 and P = 0.3929. The coeffiecient of determination (R^2) in this model was 0.9683 which coincided with adjusted- R^2 at 0.9367 and predicted- R^2 at 0.8269.

The results of ANOVA for Δ(ΔE*) from the clove extract are shown in the table 5.6.

Table 5.6: ANOVA for the response Δ(ΔE*) of the apple slices treated with the clove extract.

Source	F-Value	Prob>F	R^2	Adjusted-R^2	Predicted-R^2
Linear Model	45.49	0.0014	0.9785	0.9570	0.9427
A	90.73	0.0007			
B	0.53	0.5051			
C	79.25	0.0009			
BC	11.44	0.0277			
Lack of fit	53.15	0.1004			

Regarding to the response Δ(ΔE*), the linear model was chosen with the certain main and interactive effect. Table 5.6 showed that this model was significant on Δ(ΔE*) variable at p-value < 0.05. Like model for ΔE*, this model was contributed by the effect of clove extract concentration (A), temperature (B) and pH (C), and interactive effect between temperature and pH (BC). Each main effect was significant at p-value < 0.05 excepting the effect of temperature (B). The non-significant effect of temperature (B) was selected for the compatibility of hierarchy. For this model, the lack of fit was not significant with F=53.15 and P = 0.1004. The value of R^2, adjusted- R^2, and predicted- R^2 were conciding at 0.9785, 0.9570 and 0.9427 respectively.

5.2 Optimization

5.2.1 Optimized factor combination plan

After performing factorial design to screen the significant factors affecting on the responses, the optimization was carried out with the final purpose for sulfites replacement in the fresh-cut apples. The combination of each factor at a certain level was optimized regarding to the inhibition of the enzymatic browning expressed through ΔE^* and $\Delta(\Delta E^*)$ parameters. As the optimized factor combinations needed to be done to obtain the highest degree of the inhibition, the ΔE^* variable should be minimized while $\Delta(\Delta E^*)$ variable should be maximized as the desired responses. A number of the optimized combinations for each response from both plant extracts were shown with corresponding desirability. Desirability is an objective function to describe the extent of satisfication of response which the optimized combination can provide regarding with the desired criteria on that response. It ranges from zero to one. Desirability of zero represents a completely undesirable response value while desirability of one represents a completely desirable or ideal response value. Therefore, the desirsability should be close to one as much as possible.

Lemon balm extract

The optimized factor combinations of the lemon balm extract are shown with the predicted responses in the table 5.7 to 5.9. Then, the predicted results were verified through experimentation. Both predicted and experimental results of selected combination are compared and shown in the figure 5.1 to 5.3

The optimized combinations of lemon balm extract application to achieve the minimum ΔE*-value is shown in the table 5.7.

Table 5.7: Factor combinations for minimizing ΔE* values of the apple slices treated with lemon balm extract.

Number	Extract Concentration (%)	Temperature (°C)	pH	Predicted ΔE*	Desirability
1	0.11	38.58	9.97	3.93489	1
2	0.11	39.64	9.73	3.94767	1
3	0.11	39.19	9.86	3.92699	1
4	0.11	39.57	9.85	3.93578	1
5	0.10	39.65	9.93	3.84801	1

Based on the results from factorial design, the optimized factor combinations were proposed to five possible solutions which all of them have maximum desirability at 1. To achieve the optimum inhibition of enzymatic browning, ΔE* should be minimized. Therefore, the solution number 5 with balm extract concentration at 0.10%, temperature at 39.65 °C and pH 9.93 was selected as an optimum factor combination for this response.

The results of selected combination of lemon balm extract application to achieve the minimum ΔE*-value is shown in the figure 5.1.

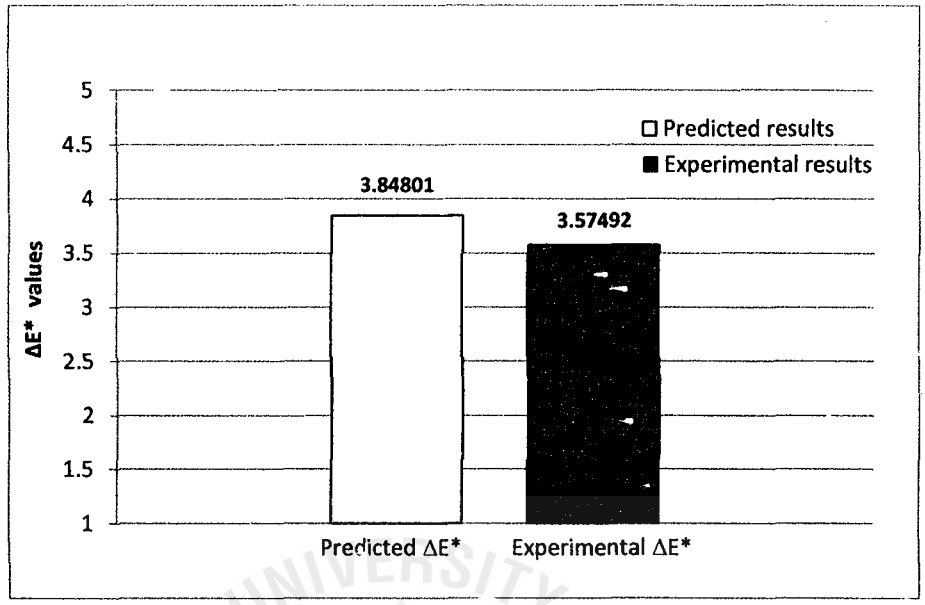

Figure 5.1: Predicted and experimental results for the optimum ΔE* response of the apple slices treated with lemon balm extract.

Figure 5.1 showed that the experimental and predicted ΔE* were positive value. But, experimental ΔE* was slightly lower than predicted ΔE*.

The optimized combinations of lemon balm extract application to achieve the maximum Δ(ΔE*)-value is shown in the table 5.8.

Table 5.8: Factor combinations for maximizing Δ(ΔE*) values of apple slices treated with lemon balm extract.

Number	Extract Concentration (%)	Temperature (°C)	pH	Predicted Δ(ΔE*)	Desirability
1	0.10	38.47	5.08	1.61371	0.918314
2	0.10	14.85	9.29	1.61370	0.918314
3	0.10	37.95	4.82	1.61370	0.918314
4	0.10	28.21	7.14	1.61370	0.918314
5	0.10	12.73	3.68	1.61370	0.918313

As there were four from five solutions of factor combination with the identically highest desirability as 0.918314, the solution which provided the maximum Δ(ΔE*) value would be selected. Therefore, the solution number 1 with balm extract concentration at 0.10%, temperature at 38.47 °C and pH 5.08 was selected as an optimum factor combination for this response.

The results of selected combination of lemon balm extract application to achieve the maximum Δ(ΔE*)-value is shown in the figure 5.2.

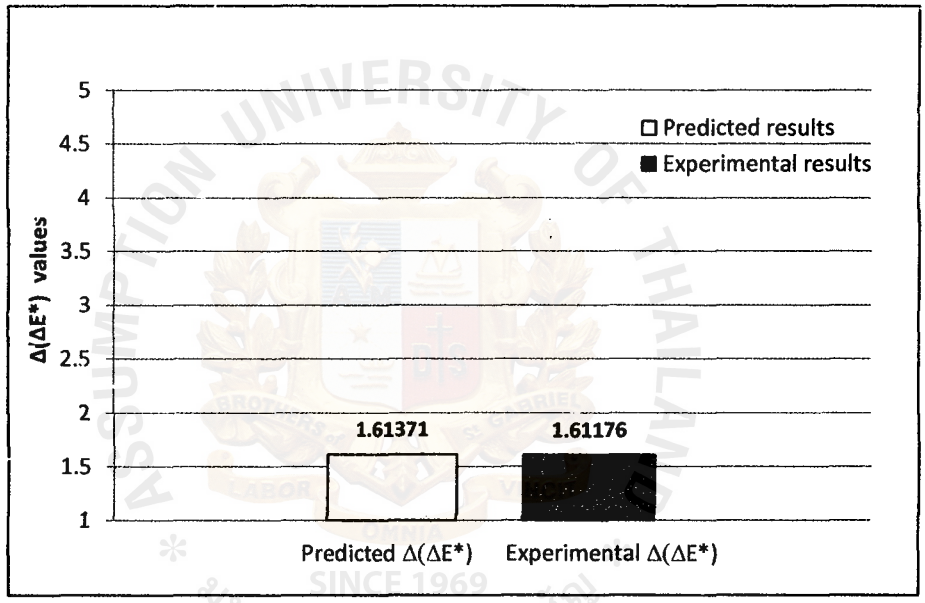

Figure 5.2: Predicted and experimental results for the optimum Δ(ΔE*) response of the apple slices treated with lemon balm extract.

Figure 5.2 showed that the experimental and predicted Δ(ΔE*) were positive value in which both values were negligibly different.

The optimized combinations of lemon balm extract application to achieve the minimum ΔE*-value and maximum Δ(ΔE*)-value simultaneously is shown in the table 5.9.

Table 5.9: Factor combination of simultaneous optimization for minimizing ΔE* and maximizing Δ(ΔE*) values of the apple slices treated with lemon balm extract.

Number	Extract Concentration (%)	Temperature (°C)	pH	Predicted ΔE*	Predicted Δ(ΔE*)	Desirability
1	0.10	39.62	9.96	3.81710	1.61370	0.958287
2	0.10	39.54	9.79	3.90110	1.61370	0.958287
3	0.10	39.95	9.58	3.95180	1.61370	0.958287
4	0.10	39.53	9.73	3.92391	1.61369	0.958286
5	0.10	39.08	9.79	3.94159	1.61369	0.958286

Among five possible optimized solutions, there were three solutions of factor combination with the identically highest desirability as 0.958287. The selection was thus made on the solution which can provide minimum ΔE* and maximum Δ(ΔE*) values. Therefore, the solution number 1 with balm extract concentration at 0.10%, temperature at 39.62 °C and pH 9.96 was selected as an optimum factor combination for these responses.

The results of selected combination of lemon balm extract application to achieve the minimum ΔE*-value and maximum Δ(ΔE*)-value simultaneously is shown in the figure 5.3.

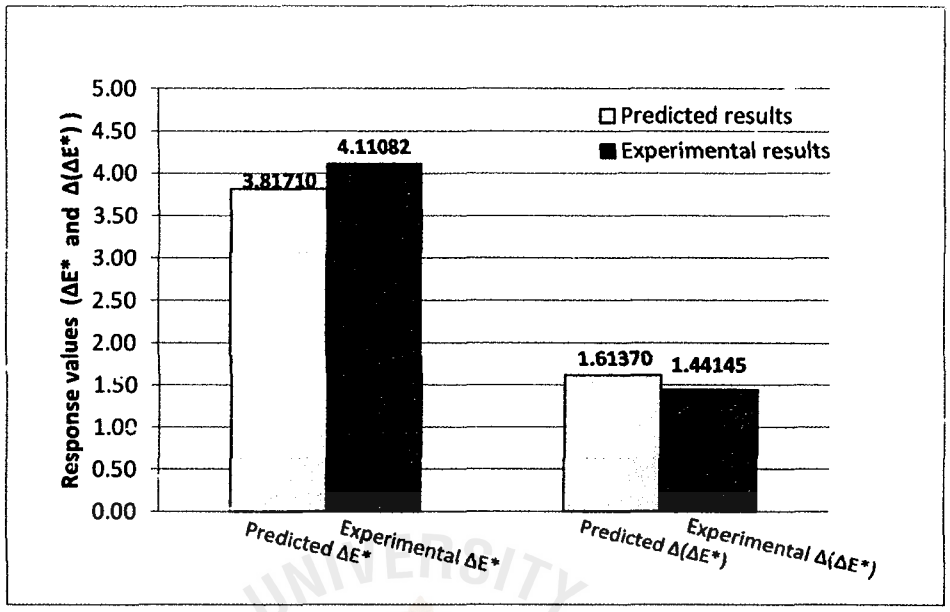

Figure 5.3: Predicted and experimental results from the simultaneous optimization for ΔE* and Δ(ΔE*) responses of the apple slices treated with lemon balm extract.

Figure 5.3 showed that the experimental ΔE* and Δ(ΔE*) were positive value which coincided with the predicted results. The experimental ΔE* was slightly higher than the predicted ΔE* while experimental Δ(ΔE*) was marginally lower than predicted Δ(ΔE*).

Clove extract

The optimized factor combinations of clove extract are shown with the predicted responses in the table 5.10 to 5.12. Then, the predicted results were verified through experimentation. Both predicted and experimental results of selected combinations are compared and shown in the figure 5.4 to 5.6.

The optimized combinations of the clove extract application to achieve the minimum ΔE*-value is shown in the table 5.10.

Table 5.10: Factor combinations for minimizing ΔE* values of the apple slices treated with clove extract.

Number	Extract Concentration (%)	Temperature (°C)	pH	Predicted ΔE*	Desirability
1	0.97	39.18	3.17	1.52788	1
2	1.00	39.17	3.01	1.48619	1
3	0.94	39.12	3.05	1.53834	1
4	1.00	37.53	3.01	1.50780	1
5	0.95	38.62	3.01	1.53691	1

As all possible combinations had the highest desirability at 1, the selection of the solution of factor combination was made upon minimum ΔE* response. Therefore, the solution number 2 was selected as an optimum factor combination for this response. According to this solution, the combination of 1% clove extract, temperature at 39.17 °C and pH at 3.01 provided the optimum ΔE* response.

The results of selected combination of the clove extract application to achieve the minimum ΔE*-value is shown in the figure 5.4.

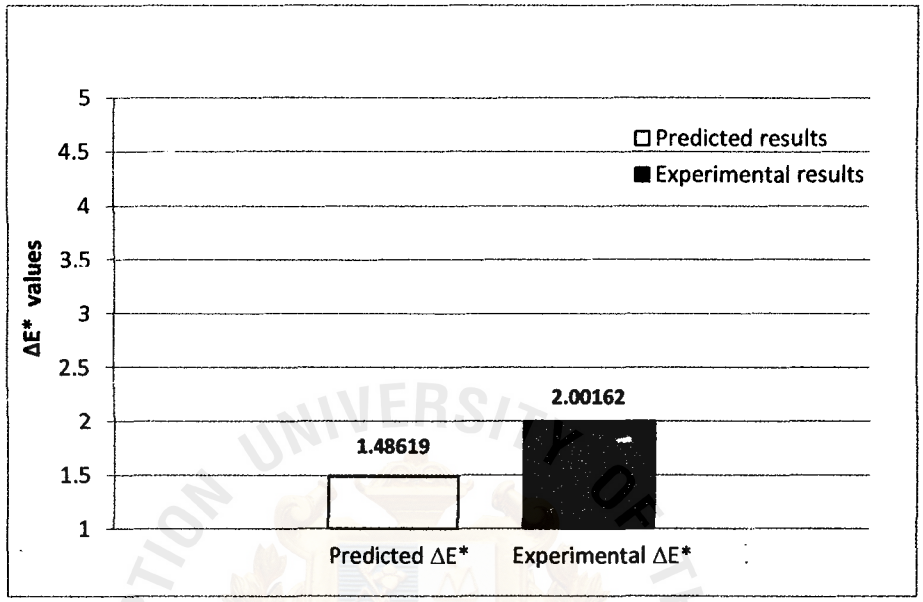

Figure 5.4: Predicted and experimental results for the optimum ΔE* response of the apple slices treated with clove extract.

Figure 5.4 showed that the experimental ΔE* were positive value which coincided with the predicted ΔE*. The experimental ΔE* was slightly higher than the predicted ΔE*.

The optimized combinations of clove extract application to achieve the maximum Δ(ΔE*)-value is shown in the table 5.11.

Table 5.11: Factor combinations for maximizing Δ(ΔE*) values of the apple slices treated with clove extract.

Number	Extract Concentration (%)	Temperature (°C)	pH	Predicted Δ(ΔE*)	Desirability
1	1.00	10.00	3.01	3.87751	0.694345
2	1.00	10.00	3.10	3.84827	0.688075
3	0.99	10.00	3.07	3.84287	0.686916
4	0.98	10.00	3.19	3.79607	0.676881
5	1.00	29.49	3.00	3.61453	0.63795

The selection of the solution of factor combination was made upon the desirability. Therefore, the solution number 1 was selected with the highest desirability as 0.694345. According to solution number one, the combination of 1% clove extract, temperature at 10.00 °C and pH at 3.01 lead to the optimum Δ(ΔE*) response.

The results of selected combination of clove extract application to achieve the maximum Δ(ΔE*)-value is shown in the figure 5.5.

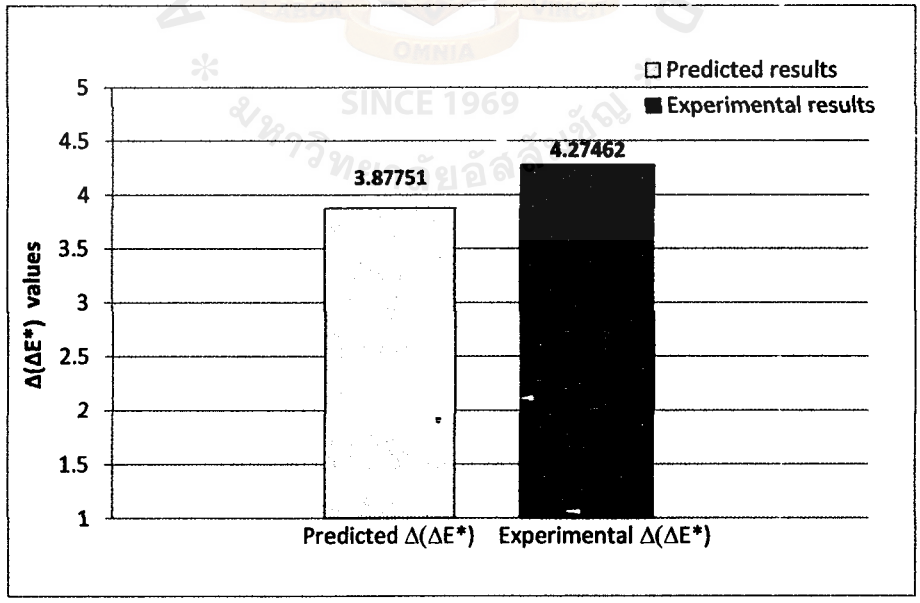

Figure 5.5: Predicted and experimental results for the optimum Δ(ΔE*) response of the apple slices treated with clove extract.

Figure 5.5 showed that the experimental Δ(ΔE*) were positive value which coincided with the predicted Δ(ΔE*). The experimental Δ(ΔE*) was slightly higher than the predicted Δ(ΔE*).

The optimized combinations of clove extract application to achieve the minimum ΔE*-value and minimum Δ(ΔE*)-value simultaneously is shown in the table 5.12.

Table 5.12: Factor combinations of simultaneous optimization for minimizing ΔE* values and maximizing Δ(ΔE*) values of apple slices treated with clove extract.

Number	Extract Concentration (%)	Temperature (°C)	pH	Predicted ΔE*	Predicted Δ(ΔE*)	Desirability
1	1.00	36.53	3.00	1.55470	3.51899	0.785788
2	1.00	39.48	3.37	1.55471	3.43371	0.774063
3	1.00	30.87	3.00	1.87808	3.59572	0.766464
4	1.00	25.86	3.00	2.1646	3.66371	0.747581
5	1.00	25.63	3.03	2.18556	3.66179	0.745281

Among five possible optimized solutions, the solution number 1 was chosen because of highest desirability (0.785788). The optimized solution number 1 suggested to use the combination of clove extract concentration at 1%, temperature at 36.53 °C and pH at 3.00 for achieving minimum ΔE* and maximum Δ(ΔE*) responses simultaneously.

The results of selected combination of clove extract application to achieve the minimum ΔE*-value and maximum Δ(ΔE*)-value simultaneously is shown in the figure 5.6.

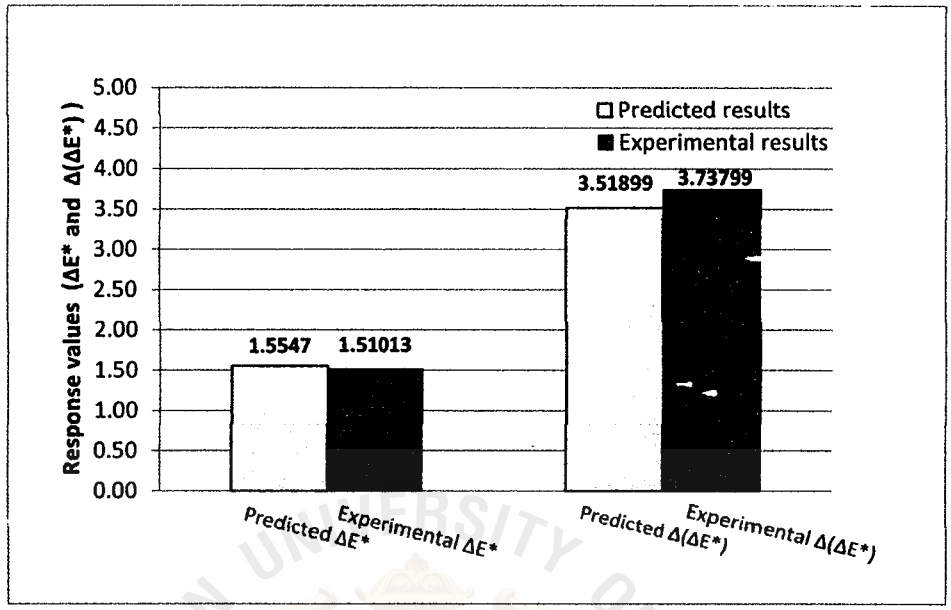

Figure 5.6: Predicted and experimental results from the simultaneous optimization for ΔE* and Δ(ΔE*) responses of the apple slices treated with clove extract.

Figure 5.6 showed that the experimental ΔE* and Δ(ΔE*) were positive value which coincided with the predicted results. The experimental ΔE* was negligibly different from the predicted one while the experimental Δ(ΔE*) was slightly higher than predicted Δ(ΔE*).

6. Discussion

6.1 Classification of the topic

At the present time, the demand for fresh-cut fruits and vegetables is rapidly increasing because of the health benefits and convenience to the consumers. Product quality is, however, limited to the enzymatic browning occurring during the processing. To prevent the reaction, vaporized SO_2 gas from sulfite is widely used as a common antibrowning agent to preserve fresh-cut fruits and vegetables. Despite their effectiveness, the use of SO_2 is restricted by the FDA due to the deleterious effects it may cause on the consumers' health. Therefore, the search for potential alternative antibrowning agents was prompted for sulfite substitution.

Until now, several physical methods and chemical agents have been proposed for inhibition of enzymatic browning [37, 47]. However, they cannot satisfy the requirements for sulfite substitution. The ideal alternatives should be safe, comparable to sulfite concerning their inhibitory effect, cost-effective, and they should not affect the sensory quality of food. Due to different possible mechanisms such as the antioxidant activities derived from phytochemicals in plant, there could be a feasibility to apply plant extract as antibrowning agents. Clove and lemon balm, for example, are herbs rich in phenolic compounds which have been investigated to provide antibrowning properties. In a previous work of screening test, it was shown that clove and lemon balm extracts had a potential on inhibiting the enzymatic browning of fresh-cut apples. Nevertheless, their effectiveness is not equivalent to sulfite. Since pH and temperature are significant factors affecting the PPO activity, the interaction between these two factors with the extract application at certain concentration may result in an improved inhibitory effect.

This present work was thus carried out to investigate the effect of concentration, temperature and pH of clove and lemon balm extracts on the inhibition of enzymatic browning in fresh-cut apples. Color measurement was carried out with using a colorimeter to determine the effect of the different treatments on enzymatic browning. In addition, the studied factors were then optimized to obtain the optimum responses concerning the enzymatic browning inhibition. The effectiveness of optimized factors would be compared among both extracts and with sulfite agent. The determination for the potential of the extracts with optimized conditions was aimed to further use as sulfite alternatives on inhibiting enzymatic browning in fresh-cut apples.

6.2 Selection of parameters for assessing the enzymatic browning

The selection of suitable parameters is required for the precise interpretation of the results. As the enzymatic browning results in the brown discoloration on the apple flesh, the color measurement and analysis can be utilized to determine the extent of discoloration and thus the degree of enzymatic browning. There is a high correlation between the color change in terms of L*-, a*- and b*-values in color space and the degree of enzymatic browning. A decrease of the L*-value is correlated with the increasing of browning [91]. Whereas the a*-value (+red to -green) increases during enzymatic browning due to the formation of brown pigments [91]. As yellowness tends to reduce after browning, the b*-value (+yellow to -blue) is decreasing along the reaction [108]. The changes in these three parameters can be combined into an appropriate single parameter called as a total color difference (ΔE^*) [52]. This would provide more convenience in the interpretation of results. This parameter is calculated as shown in section 4.2.4. An increase in ΔE^*-value is indicative of a higher degree of color change and thus a higher extent of the enzymatic browning. The ideal value for ΔE^* is zero indicating absolutely no color change. This parameter is appropriate for assessing the extent of the enzymatic browning because of its high correlation with the external visual color of fruits and vegetables [52].

To interpret the inhibitory effect on the enzymatic browning, a relative total color difference ($\Delta(\Delta E^*)$) has been defined and employed in this study. This parameter is calculated as shown in section 4.2.4. It was used for comparing the effectiveness of treatment with tap water as a control on the inhibition of the enzymatic browning. The inhibitory effect was assumed to be absent in the apples treated with water. A positive value for $\Delta(\Delta E^*)$ indicates that the degree of browning from the specific treatment is lower than that from water and thus implies its potential inhibitory effect on enzymatic browning. A higher positive value of $\Delta(\Delta E^*)$ implies a higher degree of the inhibition. In contrast, a negative value of $\Delta(\Delta E^*)$ indicates the absence of the inhibitory effect. In addition, the negative value suggests that the treatment is likely to promote the enzymatic browning instead of the inhibition. However, if $\Delta(\Delta E^*)$ is obtained as zero value, this indicates that the degree of browning of the apples treated with the extracts and water control occurs at the same extent. In other words, the treatment does not provide the inhibitory effect. The desirable $\Delta(\Delta E^*)$ value related to browning inhibition should be as positive as possible . In addition, ΔE^*

of water control represented as $\Delta E_{\text{water control}}$, which is used for the calculation of the $\Delta(\Delta E^*)$-value, is essential because it can describe the natural variation in apples.

6.3 Factors affecting the variation in degree of enzymatic browning

From the results of apple slices treated with water as control shown in the appendix A (table 9.4), there was an obvious variation in degree of browning and value of $\Delta E_{control}$ ranging from 3.09 to 11.00. This variation occurred presumably due to the natural variation of chemical compositions (especially phenolic compounds and ascorbic acid) and enzyme PPO found in apples itself. This variation is considerably uncontrollable factors that can cause the deviation in the results. Regarding PPO enzyme, its content is reported to fluctuate among apple fruits because of the differences in the growing conditions [30]. Besides enzyme content, PPO activity and its physiological properties such as temperature and pH optimum, and thermal stability has been also reported to fluctuate with maturity, substrates, and growing condition [66]. For example, PPO activity is generally higher in young fruits [66]. However, a fluctuation of PPO activity in apples was mostly found during storage period [66]. The variation in PPO activity was also detected in the tissue level. *Nicolas, et al. (1994)* reported that PPO activity in peel is higher than in cortex of apples whereas the others proposed opposite information [66]. Consequently, this could also effect on the variation in ΔE^*-value of the treatment [30, 47].

Concerning the chemical composition, the content of phenolic compounds and ascorbic acid can naturally vary among apples despite the same variety. Chlorogenic acid, which is the most favorable substrate for apple's PPO, is found to vary from 5 – 51 mg/100 g fresh weight basis [60, 66]. The variation of phenolic composition is found in both subcellular and tissue level [66]. Apart from phenolics, there is the considerably seasonal variation in the ascorbic acid content in which the more ripened fruit contains a higher amount of ascorbic acid [54]. Since this acid can act as the inhibitor in the enzymatic browning, the degree of browning is also varied with the ripeness [53]. The natural variation in PPO, phenolic compounds and ascorbic acid content is greatly affected by the degree of ripeness, stage of maturity, climacteric factors, type of fertilizer used, antibiotic and insecticide application, and post harvest storage conditions [60, 66]. The natural variation of these factors can induce different tissue sensitivities to enzymatic browning

[66]. However, we would try to minimize these natural variations by using the same apple cultivar from the same source which provided the newly fresh apples. Besides, all the apples used for the treatment would be stored no longer than one week.

Apart from the variability on PPO and chemical compositions, the fluctuation in responses could be affected by an instability of b* parameter. There is a frequent report on the large fluctuation of b*-values [22, 91]. From the appendix (table 9.3), there was an apparent variation in b*-values ranging from -0.61 to 12.83.This fluctuation was believed to occur as a result of the production of purple pigments from the reaction between intermediates in the enzymatic browning [22]. This reaction step cannot be prevented by antibrowning agents, thus the fluctuation of b*-values is always present but with a different extent [84]. The fluctuation of the parameter b* is one factor affecting the variation in all three parameters: ΔE^*-value, $\Delta E_{control}$ and $\Delta(\Delta E^*)$- value. However, the extent of this effect on each parameter is still not known.

6.4 Interpretation of the infiuence of factors on enzymatic browning

The interpretation will be carried out on the effect of lemon balm extract and subsequently of clove extract.

Lemon balm extract

Regarding the treatments with lemon balm extract, the interpretation will be carried out first for the response ΔE^* and then $\Delta(\Delta E^*)$ value, respectively.

Interpretation of the model for the response ΔE^* from the factorial design

The ANOVA Table (Table 5.3) showed that the model for the response ΔE^* had a high coefficient of determination (R^2) of 0.9879. This means that 98.79% of the variation in ΔE^* can be described by the model in which the variation was resulted from the effect of significant factors. The remaining 1.21% of the variation, which cannot be explained by the model, might be

resulted from the natural variation as mentioned in the section 6.3. Table 5.3 also showed that the values for R^2, Adjusted-R^2, and Predicted-R^2 were proximate to each other, indicating the reliability of the model. As the lack of fit was non-significant ($p>0.05$), this model practically fit with the data.

Interpretation of the effects of the factors on the response ΔE*

The ANOVA Table (Table 5.3) also showed that the factors that significantly affect the ΔE* of apple slices treated with lemon balm extract were extract concentration, temperature and pH without significant interactive effects. The effect of each factor has been illustrated in figure 6.1 to 6.3.

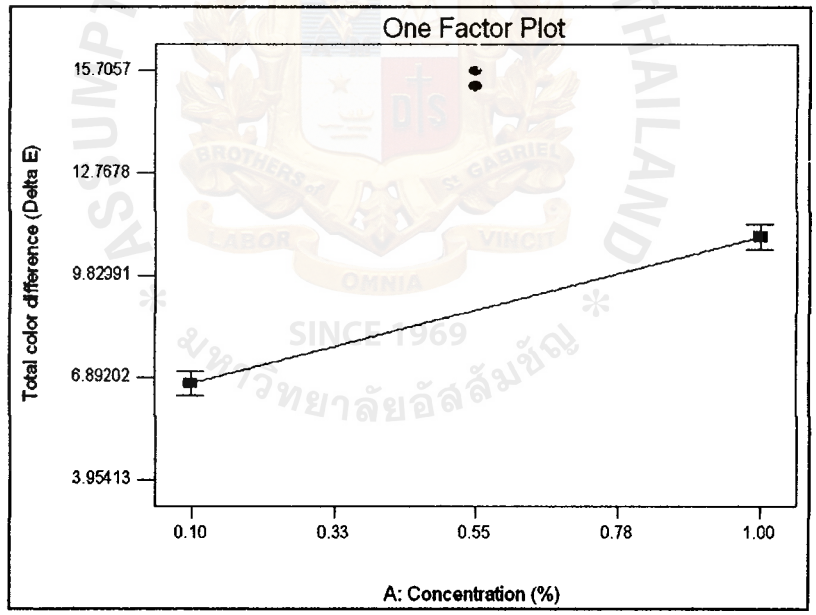

Figure 6.1: Effect of lemon balm extract concentration on a total color difference (ΔE*) of treated apple slices (center point (•) = treatment at 0.55% extract concentration, 25°C, pH 5.5).

The evaluation on factor whether it significantly affects on the response can be done by considering the "least significant difference" (LSD) bars. This LSD bar locates at the both ends

of the linear plot. The square point at the middle of the bar represents the mean value of the response at the certain level of factor. The superimposition between LSD bars confirms the lack of significant difference in the response and thus non-significance in the factor effect. Figure 6.1 showed that there was obviously no overlapping between LSD bars. It also showed that the increase in concentration of lemon balm extract from 0.1% to 1% caused a significant increase in the degree of browning expressed through ΔE^*.

Theoretically, the main phenolic compound in lemon balm is rosmarinic acid which could provide the antioxidant activity to inhibit the enzymatic browning [20]. However, the lemon balm leaf also contains hydroxycinnamic acids counted as 11.29% which include the caffeic acid and chlorogenic acid that act as PPO substrates [64, 101]. Lemon balm contains both type of phenolic compounds which can act as PPO substrates or inhibitors [20]. The former and latter phenolics can be called as phenolic substrates and phenolic inhibitors respectively. Although the rosmarinic acid is predominant in lemon balm, its amount together with other phenolic inhibitors might not be comparable to the amount of phenolic substrates derived from both extract itself and apple. Besides, the phenolic inhibitors are normally effective when their amount is much higher than the PPO substrates [50]. Furthermore, as the lemon balm extract also contains cinnamic and benzoic acid, their amount would be increased at a higher extract concentration [20].When these two aromatic carboxylic acids are present at a high concentration, they can be converted to caffeic acid which is considered as PPO substrates [85]. Hence, when the extract concentration increases, the amount of phenolic substrates is much higher than phenolic inhibitors at the greater extent. For this reason, the higher degree of browning with the higher ΔE^* would be obtained at a higher concentration of lemon balm extract. The degree of browning and ΔE^* value at 0.1% concentration was, thus, lower due to a lower amount of phenolic substrates.

In addition, the increase in ΔE^* at 1% concentration might be due to the effect of tissue damage [55]. The study from *Lu, et al. (2007)* showed that the tissue damage is one cause of a decrease in L*-value when the concentration of antibrowning agents was increased [55].

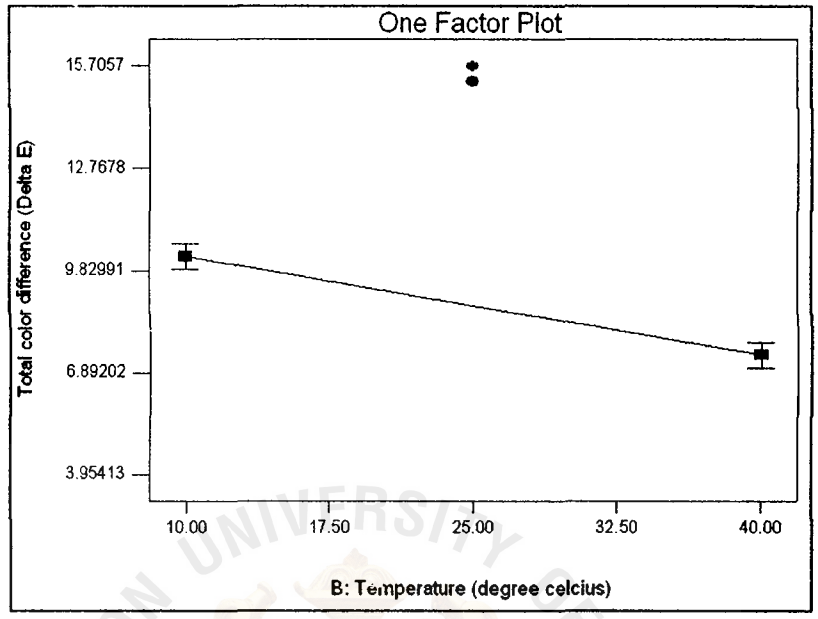

Figure 6.2: Effect of temperature of lemon balm extract on a total color difference (ΔE*) of treated apple slices (center point (•) = treatment at 0.55% extract concentration, 25°C, pH 5.5).

Figure 6.2 showed a significant decrease in ΔE* with an increasing temperature from 10°C to 40°C ($p<0.05$). Obviously, it showed that the degree of browning expressed through ΔE* at 40°C was lower than at 10°C. The decreasing degree of browning at 40°C could be due to the fact that PPO activities are reduced and disrupted when temperature increases away from the optimum (25°C), [67]. The results have also agreed with other studies. For instance, *Oms-Oliu, et al. (2006)* reported that PPO activities primarily declined at 35-45°C depending on the enzyme source and substrate [67]. Besides, *Kunnikar, et al. (2009)* reported that the percentage of PPO inhibition at 40°C is higher than at 25°C [46]. The explanation of the PPO inhibition might be due to that the moderate heat produced at 40°C could cause a partial physiological modification of enzyme. To illustrate, it could causes the disruption of complex covalent and non-covalent structures resulting in the interruption of PPO activities [48, 53]. Apart from the PPO inhibition, the effect of temperature changes might have an influence on the phenolic substrates. The configuration of the phenolic substrates may be disturbed or partially changed at 40°C so the binding of substrate at active site would be obstructed [46]. Conclusively, the decreasing degree

of browning expressed through ΔE* at temperature 40°C is presumably due to the alteration of the phenolic substrates' structure and the inhibition of PPO activities. On the other point of view, an increase in temperature builds up a heat which could also initiate the non-enzymatic browning reaction such as maillard reaction [40]. The extent of browning depends on a degree of temperature and heating time [40]. Since temperature at 40°C produced a moderate heat and the dipping time was only 10 minutes, this would not be sufficient to induce the maillard reaction. Therefore, this aspect was not taken into account for consideration.

Temperature at 10°C was much lower than the optimum temperature of PPO so the inhibition of enzymatic browning might also be taken place. When the temperature was decreased to 10°C, the kinetic energy of the reactants was reduced. This further contributed to the decrease of mobility and effective collisions which are essential for forming the enzyme-substrate complexes and their products [58]. However, the inhibition efficiency at 10°C may not be as comparable as at 40°C because the effect of temperature at 10°C did not directly modify the structure of enzyme or substrate as temperature at 40°C did. In addition, *Martinez, et al. (1995)* reported that the PPO activities are significantly retarded at temperature below 4°C [53]. Therefore, the temperature at 10°C might not be low enough to provide the significant inhibition. These are the reasons that the degree of browning expressed through ΔE*-value at 40°C was significant lower than at 10°C.

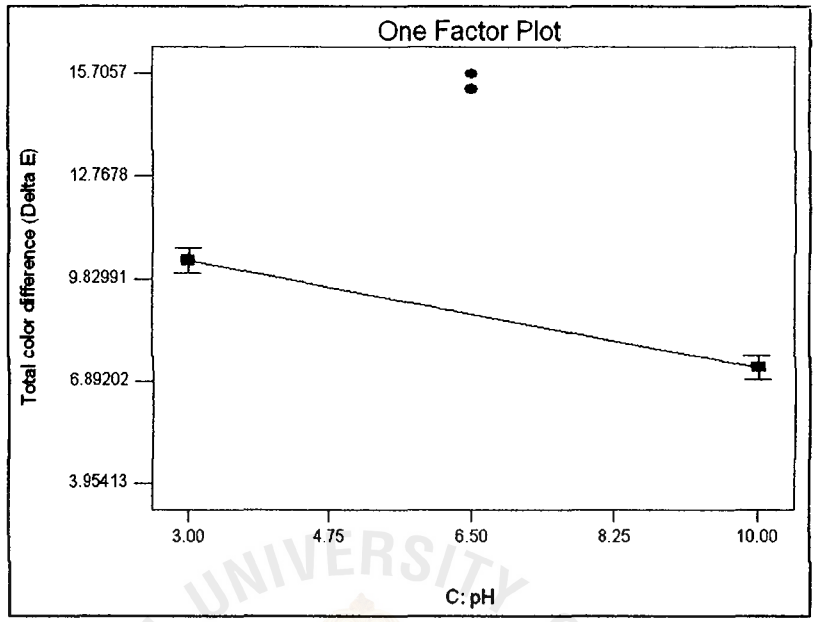

Figure 6.3: Effect of pH of lemon balm extract on a total color difference (ΔE*) of treated apple slices (center point (•) = treatment at 0.55% extract concentration, 25°C, pH 5.5).

Figure 6.3 showed that an increase in pH from 3 to 10 has lead to a significant decrease in ΔE*-value. This implied that the degree of enzymatic browning at acidic pH was greater than alkaline pH for the lemon balm extract. As mentioned before, lemon balm extract contains high amount of hydroxycinnamic acids acting as the PPO substrates [14, 58]. The important carboxylic structure has facilitated these substrates to bind at the active site of the enzyme [58]. As pH 10 was obtained by addition of NaOH solution, the hydroxide ion from NaOH can induce the dissociation of the carboxylic group of hydroxycinnamic acids resulting in an alteration of their configuration. Subsequently, the affinity of these substrates to PPO enzyme was lowered so the ability to promote browning of these phenolic substrates was reduced at pH 10. This was supported by the study of *Rocha, et al. (2001)* who reported that the apparent *K*m of chlorogenic acid for PPO has increased above pH 5.0 [80]. On the other hand, when the pH of the extract is acidic, the hydroxycinnamic acids are still present in the un-dissociated form [80]. The un-dissociated hydroxycinnamic acids at pH 3 can normally function as the substrates, so the sensitivities to the enzymatic browning at pH 3 was higher than at pH 10. Moreover, the extract at pH 3 might not be sufficient to provide the effective inhibition on PPO since this enzyme is

completely inactivated at pH below 3 [61, 63]. These might be the reasons why the degree of browning expressed through ΔE* was significantly decreased when pH of the extract was increased from 3 to 10.

Interpretation of the model for the response Δ(ΔE*) from the factorial design

The ANOVA Table (Table 5.4) showed that the coefficient of determination (R^2) of the statistical model for the response Δ(ΔE*) was 0.9741. This can be implied that 97.41% of variation in Δ(ΔE*) can be described by the model while the remaining was probably due to unpredicted factors as mentioned in section 6.3. Table 5.4 also showed that the values for R^2, Adjusted-R^2, and Predicted-R^2 corresponded to each other, indicating the reliability of the model. Lack of fit was not significant ($p>0.05$), thus, this model practically fit with data.

Interpretation of the effects of the factors on the response Δ(ΔE*).

The ANOVA Table (Table 5.4) also showed that only the concentration of lemon balm extract significantly affected Δ(ΔE*)-value of treated apple slices. The effect of extract concentration on Δ(ΔE*) has been illustrated in figure 6.4.

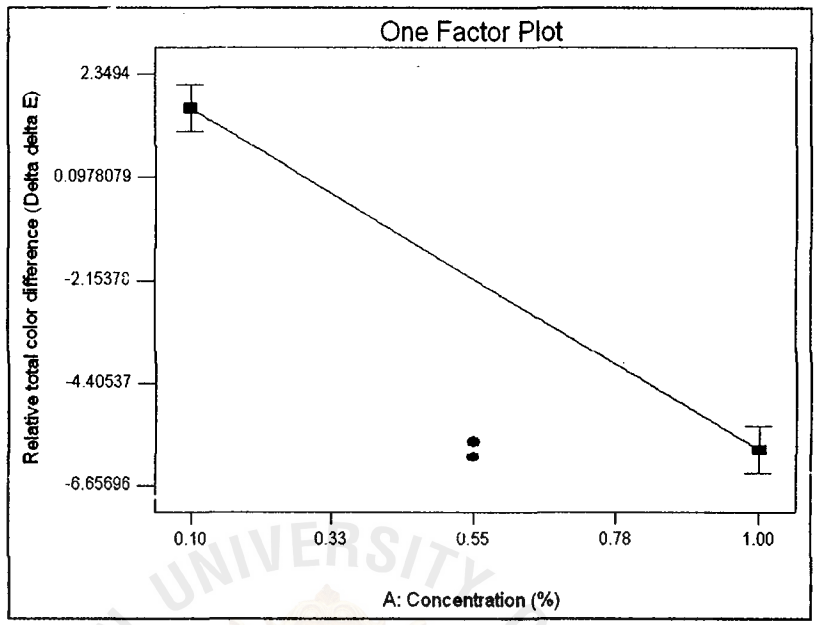

Figure 6.4: Effect of lemon balm extract concentration on a relative total color difference ($\Delta(\Delta E^*)$) of apple slices (center point (•) = treatment at 0.55% extract concentration, 25°C, pH 5.5).

Figure 6.4 showed that an increase of lemon balm extract concentration from 0.1 to 1% had lead to a significant decrease in $\Delta(\Delta E^*)$ from positive to negative value. The presence of both positive and negative value of $\Delta(\Delta E^*)$ suggested that the lemon balm extract could provide the inhibition as well as the promotion on the enzymatic browning. Concerning the inhibitory effect, it was possibly ascribed to the antioxidant activities and the disruption of PPO activities by phenolic compounds in the lemon balm extract. For the antioxidant activities, it is mainly provided by rosmarinic acid [20]. The antioxidant activities of this phenolic could inhibit the enzymatic browning by donating a hydrogen atom to neutralize free radicals which can interact with a substrates to induce the enzymatic browning as already explained in the section 3.4 (*Potential inhibitory effect of phenolic compounds on enzymatic browning*) [20]. Apart from antioxidant activity, the extract contains other phenolics which act as PPO inhibitors. First, cinnamic acid and its derivatives such as *p*-coumaric, ferulic acid, and caffeic acid in the balm extract have been found to be PPO inhibitors in various fruits including apples [14, 29, 53, 104]. The undissociated carboxylic groups enable these acids to complex with copper at the active site and

perform the inhibition [14, 58]. Second, lemon balm also contains benzoic acid and its derivatives especially protocatechuic whose the inhibitory effect has been reported on the PPO of mushroom [46, 109]. Third, condensed tannin presenting in lemon balm has been found to inhibit the activity of PPO through bonds formation at the active site [9, 32]. Apart from phenolic acids, lemon balm also consist of flavonoids especially flavanols and flavanones but in a few amount as 0.51% in leaves [14, 20, 68]. Their inhibition mechanism is presumably due to the chelation of copper in the enzyme [68]. Conclusively, these phenolics mentioned above synergistically contributed to the antibrowning activities of lemon balm extract and thus the positive $\Delta(\Delta E^*)$ values.

On the other hand, the negative $\Delta(\Delta E^*)$ values from figure 6.4 implied that the extract could also promote the enzymatic browning. Apart from phenolic inhibitors, lemon balm also contains a substantial amount of phenolic substrates which provide the promoting effect on the enzymatic browning. The content of phenolic substrates derived from extract itself and apples might be higher than phenolic inhibitors as explained earlier in "the effect of lemon balm extract concentration on ΔE^*" (figure 6.1).

Regarding to the results from figure 6.4, it can be interpreted that when the extract concentration increased, the inhibitory effect on enzymatic browning compared with water was successively reduced until completely depleted. After depletion of inhibitory effect, the increase in extract concentration would promote the enzymatic browning. This assumption can be clarified with below statements. The phenolic substrates derived from lemon balm extract at 0.1% concentration were still present at low content. Therefore, at this concentration, the inhibitors from the extract might still be able to perform the inhibition on the enzymatic browning. This would be one reason that $\Delta(\Delta E^*)$ at 0.1% was still relatively high when compared with that at 1% concentration. Nevertheless, when the extract concentration was increased, the content of phenolic substrates was also increased to not only promote the enzymatic browning but also to reduce the activity of the inhibitors [50]. The response $\Delta(\Delta E^*)$ has been gradually decreased with the increasing extract concentration until the amount of phenolic substrates was sufficiently high to mask the inhibitory effect from phenolic inhibitors. Then, a further increase in extract concentration would promote the enzymatic browning instead. Finally, negative values of $\Delta(\Delta E^*)$ were obtained.

Considering the results of the response ΔE^* from figure 6.1, it showed that the increase of lemon balm extract concentration from 0.1 to 1% caused the significant increase in ΔE^* ($p<0.05$). The significant increase in ΔE^* can be another explanation to the decrease in $\Delta(\Delta E^*)$ since both parameters are negatively correlated.

Clove extract

Regarding the treatments with clove extract, the interpretation will be carried out first for the response ΔE* and then Δ(ΔE*) value, respectively.

Interpretation of the model for the response ΔE* from the factorial design.

The ANOVA from table 5.5 showed that the model for the response ΔE* had a coefficient of determination (R^2) of 0.9683. This indicated that 96.83% of the variation in the response ΔE* can be explained by the model while the remaining of the variation, which was unexplainable, might be ascribed to the natural variation mentioned in section 6.3. Since R^2, Adjusted-R^2, and Predicted-R^2 values from table 5.5 were proximate to each other, this indicated the reliability of the model. However, Predicted-R^2 was slightly lower than the other two values. As lack of fit was not significant ($p>0.05$), this model practically fit with data.

Interpretation of the effects of the factors on the response ΔE*.

The ANOVA from table 5.5 showed that the effect of concentration, temperature and pH, and interactive effect between temperature and pH of the clove extract were significant factors affecting the response ΔE*. The effect of each factor has been illustrated in figure 6.5 to 6.8.

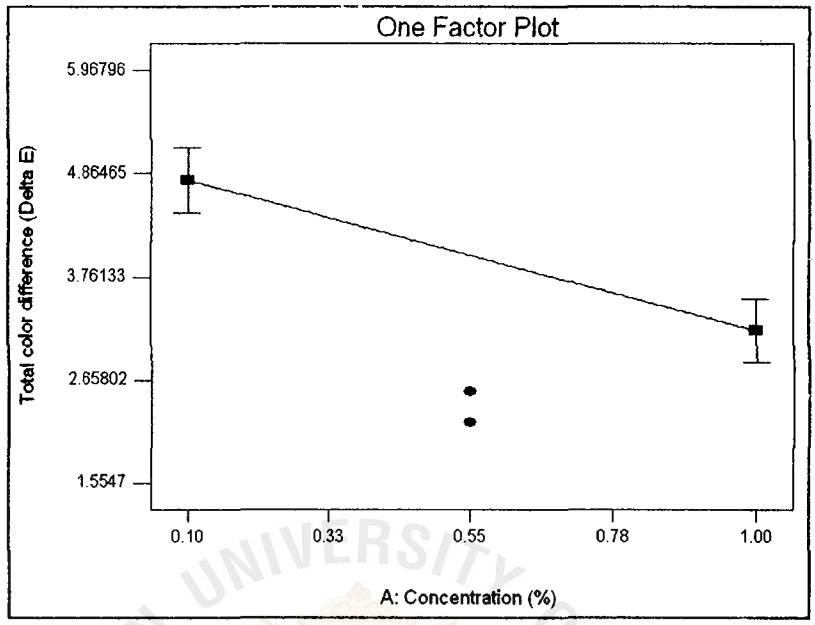

Figure 6.5: Effect of clove extract concentration on a total color difference (ΔE*) of apple slices (center point (•) = treatment at 0.55% extract concentration, 25°C, pH 5.5).

Figure 6.5 showed that an increase in concentration of clove extract from 0.1% to 1% had lead to a significant decrease in ΔE*-value. In other words, it had lead to a decrease in degree of browning. The decrease in the degree of browning could be attributed to the increasing inhibitory effect from chemical substances contained in the clove extract. There are several compounds in the clove extract responsible for the inhibition of enzymatic browning. First, the inhibitory effect could be attributed to antioxidant activities of eugenol and eugenol acetate derived from clove oil [71, 89]. Second, clove also contains many types of flavonols including quercetin, kaempherol, rhhamnetin and flavonol glycosides which have been reported to inhibit the PPO activities [16, 69, 109]. However, these flavonols are defined as the weak PPO inhibitors [16]. Third, like lemon balm, clove contains tannin and benzoic acids which act as PPO inhibitors [16]. The major benzoic acids in clove are gallic acid, vanillic acid, salicylic acid and 2,4 dihydroxy benzoic acid which could inhibit the PPO activites by exerting a copper chelating mechanism [16, 29, 49, 69]. Forth, apart from phenolic compounds, clove also contains benzaldehyde whose aldehyde group is known to inhibit PPO [16, 49]. The major inhibitory action of benzaldehyde is accomplished on the ground of its ability to form a Schiff base with the primary amino group of

the enzyme [16]. Lastly, the ascorbic acid in clove also has an important role in inhibiting the enzymatic browning reaction as already described in the section 3.4 (Theoretical background). However, the inhibitory effect of clove extract would be influenced by the solubility of those compounds in extract itself. Their soluble properties are different in which eugenol is soluble in oil and alcohol whereas phenolic acids and ascorbic acid is preferably soluble in water. Conclusively, when the clove extract concentration was increased, the content of various inhibitors mentioned above also increased [43, 71]. Consequently, this could promote the inhibition on the enzymatic browning leading to a significant decrease of ΔE* values.

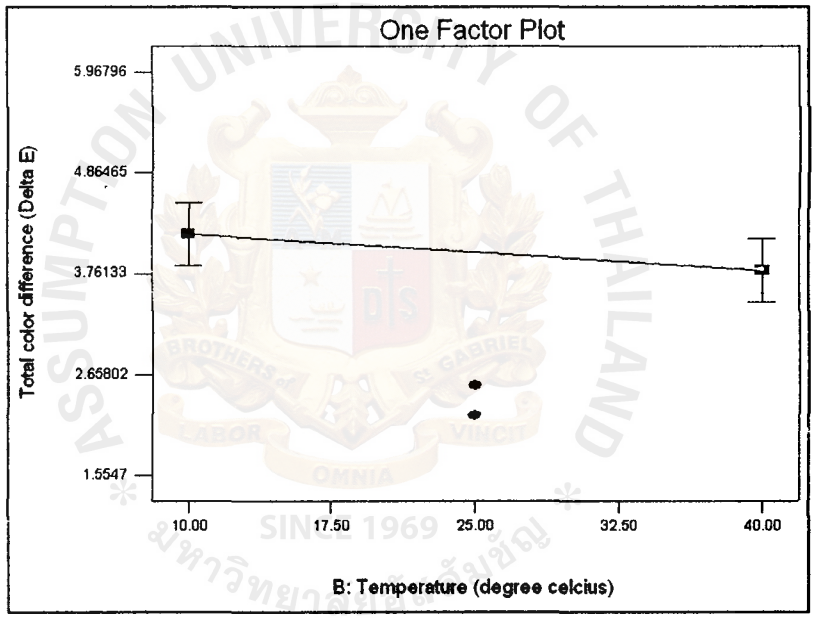

Figure 6.6: Effect of temperature of clove extract on a total color difference (ΔE*) of apple slices (center point (•) = treatment at 0.55% extract concentration, 25°C, pH 5.5).

Figure 6.6 showed that an increasing temperature from 10°C to 40°C did not result in a significant change in the response ΔE*. However, there was a significant interactive effect between temperature and pH as shown in table 5.5. Therefore, the factor of temperature had to be included in the model for the compatible hierarchy. Clove extract contains a substantial amount of various browning inhibitors cooperatively providing the inhibitory action, so the effect of

temperature is possibly masked by the considerable inhibitory strength of extract itself [43, 49, 104]. This might be one explanation to the non-significant effect of clove extract temperature.

Comparing with the results of lemon balm extract (figure 6.2), the increase of lemon balm extract temperature up to 40°C caused the significant decrease in ΔE*. This implied that generally extract temperature should provide a significant effect on the browning inhibition. However, the variations of optimum temperature and thermal stability of PPO in apple might be one determinant which contributes to the difference in the results between clove and lemon balm extract [59]. To clarify, clove extract at 40°C did not provide the significant inhibition because the apples used in the clove treatment might have the higher PPO thermal stability than those used in the balm treatment. On the other hand, the effect of lemon balm extract's temperature was significant on ΔE* might be due to the reason that the inhibitory effect of chemical substances in lemon balm might not be high enough to mask the effect of temperature like clove extract did.

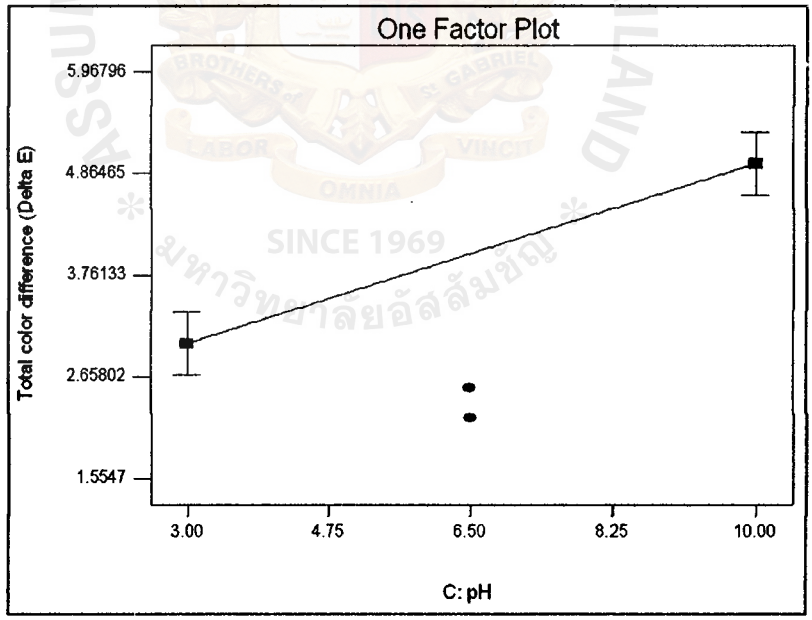

Figure 6.7: Effect of pH of clove extract on a total color difference (ΔE*) of apple slices (center point (•) = treatment at 0.55% extract concentration, 25°C, pH 5.5).

Figure 6.7 showed that the increase from pH 3 to pH 10 had lead to a significant increase in ΔE*-value. This implied that the degree of browning at pH 3 was significantly lower than at pH 10. This outcome could be explained through the following explanation. *Mccord, et al. (1983)* reported that PPO activity is more preferentially reduced at acidic pH than at alkaline pH [61]. He also suggested that the protonation of free carboxylic group in the enzyme molecule by acid can result in electrostatic repulsive forces [61]. These forces can aggravate PPO activity by partially distorting the tertiary structure of the enzyme [61, 102]. In addition, *Altunkaya, et al. (2011)* reported that below pH 4 could cause the looser binding of copper at the active site [2]. This loosing further facilitates the copper chelation which finally leads to a decrease in PPO activities [2, 59]. Although most results from literatures reported that PPO is usually inactivated below pH 3, some studies have confirmed that the inactivation of PPO can also be achieved at pH 3[6, 61, 63, 95]. This contradiction might be due to the fact that optimum pH of PPO in apples can be varied in accordance with a natural variation as mentioned in section 6.3.

Besides, chloride ion (Cl^-) from HCl added during acidification could inhibit the PPO activities by reacting with a positively charge of imidozole group at the active site of enzyme [95]. The inhibitory effect on PPO of this halogen is dependent on pH in which a lower pH promotes a greater effect. Moreover, the inhibitory effect from substances contained in clove is promoted at acidic. The acid protonation facilitates the function of most of PPO inhibitors [29, 58, 63]. Therefore, the increase of ΔE* at pH 10 might be assumed as a result of the fact that alkaline pH does not enhance the inhibitory effect of PPO inhibitors. In addition, the increase of ΔE* at pH 10 might be attributed to the rapid non-enzymatic browning of substrates which is normally induced at alkaline pH [63, 80].

In contrast, ΔE*of apple slices treated with lemon balm extract at pH 10 was significantly lower than at pH 3. Lemon balm contains a considerable amount of phenolic substrates, but most phenolics presenting in clove are PPO inhibitors [20, 49]. Therefore, the effect of pH is more influential on the function of PPO inhibitors in clove extract. On the one hand, it more influences on the function of phenolic substrates in lemon balm extract as earlier explained in "the effect of pH of lemon balm extract on ΔE*" (figure 6.3). Thus, the results of pH effect on ΔE* value of clove extract has contradicted to that of the lemon balm extract.

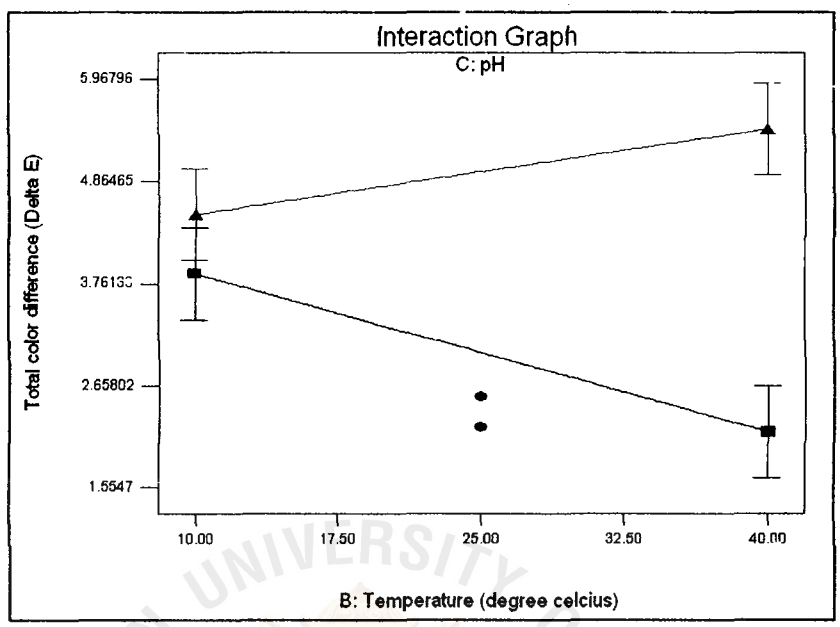

Figure 6.8: Interactive effect between temperature (B) and pH (C) (■ = pH 3, ▲ = pH 10) of clove extract on a total color difference (ΔE*) of apple slices. (center point (•) = treatment at 0.55% extract concentration, 25°C, pH 5.5).

The presence of interactive effect was found from the evaluation of graph profile (figure 6.8). If two linear lines in the plot are not parallel to one another, it can be interpreted that there is an interactive effect between the two factors. The plot in figure 6.8 showed nonparallel lines which indicated the presence of the interactive effect between temperature and pH. In addition, the results of statistical analysis in table 5.5 also confirmed this interactive effect. This means that temperature and pH cooperatively interacted to affect the response ΔE*. From the figure 6.8, it showed that when temperature increased from 10°C to 40°C, ΔE*-value significantly decreased at pH 3 (■), but remained constant at pH 10 (▲).Therefore, the effect of temperature on the changing of ΔE* and the browning inhibition also depended on pH. However, the effect of temperature was significant only at pH 3. This can be concluded that temperature at 40°C combined with low pH (pH 3) gave the best inhibitory effect by presenting the lowest ΔE*.

Theoretically, temperature has an effect on the dissociation of acid and basic molecules in a solution [27]. The increased kinetic energy, caused by an increasing temperature, can promote the dissociation rate whereas a decreasing temperature provides the opposite effect [27].

Therefore, if temperature is increased to 40°C, the dissociation of acid or base will be also increased. On the other hand, it will be decreased if temperature is decreased to 10°C. The change in the dissociation ability of acid or base has further affected on the concentration of hydrogen ion (H^+) and hydroxide ion (OH^-) respectively [27].

Figure 6.8 showed that at pH 3(■), degree of browning expressed through ΔE* at 40°C was significantly lower than at 10°C. This could be because the effect of temperature at 40°C can promote the dissociation of acid molecules into hydrogen ions (H^+) [27]. Subsequently, the H^+ concentration was increased into a level which can severely inactivates PPO activities [6, 27, 95]. On the other hand, the acid dissociation rate was reduced after temperature was decreased to 10°C, so H^+ concentration could be reduced into a lower level which might be able to perform only the partial inhibition of PPO activities [6, 27, 95].

Noticeably, at any temperature, ΔE* at pH 10 remained higher than that at pH 3. Moreover, considering at pH 10 (▲), ΔE* was not significantly different when temperature changed. Although hydroxide ion (OH^-) concentration would be increased at a rising temperature up to 40°C due to the enhanced dissociation rate of basic molecules, this did not promote the higher extent of the browning inhibition. These outcomes could be explained by the reason that the enzymatic browning is preferentially inhibited at acidic rather than at alkaline condition [61].

Interpretation of the model for response Δ(ΔE*) from the factorial design.

The ANOVA Table (Table 5.6) showed that the coefficient of determination (R^2) of the statistical model for Δ(ΔE*) response was 0.9785 which also corresponded with Adjusted-R^2 and Predicted-R^2. This can be implied that 97.85% of the variation in Δ(ΔE*) can be described by the model while the remaining of variation, which was unexplainable, might be ascribed to the natural variation mentioned in section 6.3. As a lack of fit was not significant ($p>0.05$), this model practically fit with data.

Interpretation of the effects of the factors on the response Δ(ΔE*).

The ANOVA Table (Table 5.6) showed that the effect of concentration, temperature, pH, and interactive effect between temperature and pH of the clove extract were significant factors affecting the response Δ(ΔE*). The effect of each factor has been illustrated in figure 6.9 to 6.12.

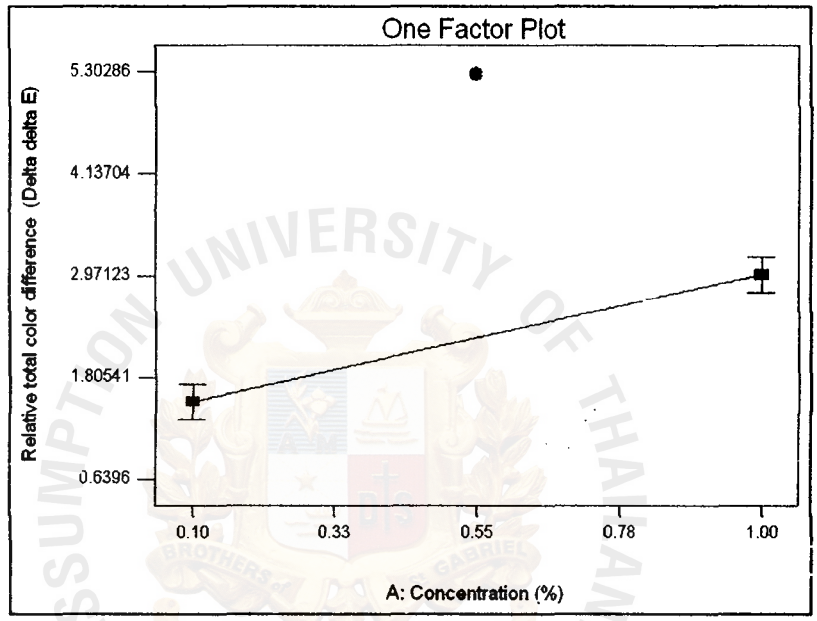

Figure 6.9: Effect of clove extract concentration on relative total color difference (Δ(ΔE*)) of apple slices (center point (•) = treatment at 0.55% extract concentration, 25°C, pH 5.5).

Figure 6.9 showed that when the concentration of the clove extract increased from 0.1% to 1%, Δ(ΔE*) significantly increased. This implied that the inhibitory effect was more efficient with an increasing extract concentration. The reason is that when the extract concentration increased from 0.1% to 1%, the content of chemical substances, which can perform the inhibition on the browning, also increased in a positive correlation.

This implication coincided with the interpretation described in "the effect of clove extract concentration on ΔE*" (figure 6.5). Since Δ(ΔE*) is negatively correlated with ΔE* parameter,

the significant decrease of ΔE*, as shown in figure 6.5, could contribute to the significant increase of Δ(ΔE*) in figure 6.9 if the variation of $\Delta E_{\text{water control}}$-value is insignificant.

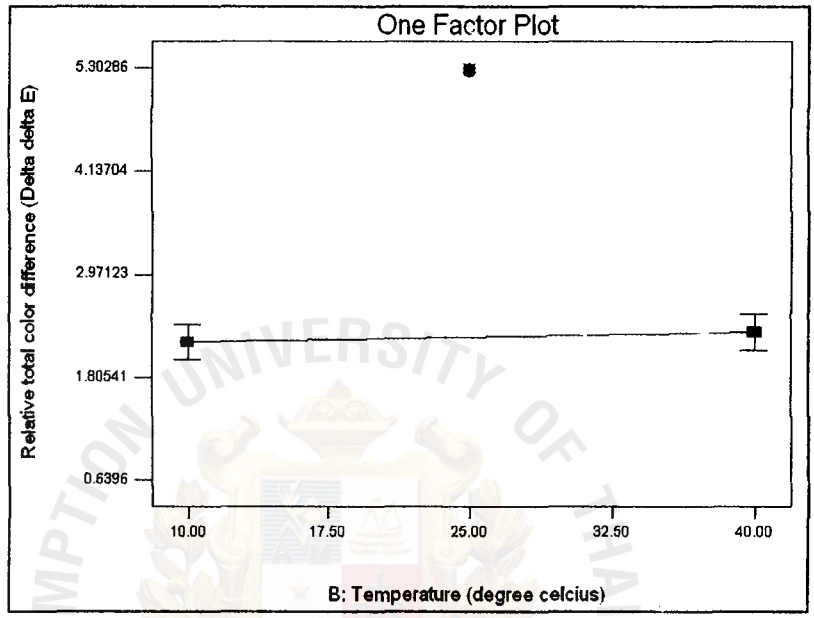

Figure 6.10: Effect of temperature of clove extract on relative total color difference (Δ(ΔE*)) of apple slices (center point (•) = treatment at 0.55% extract concentration, 25°C, pH 5.5).

Figure 6.10 showed that the increasing temperature from 10°C to 40°C did not create a significant difference in response Δ(ΔE*). This can be concluded that the inhibitory effect on the enzymatic browning at temperature between 10°C to 40°C was not significantly different ($p>0.05$). The effect of temperature may be masked by the considerable inhibitory effect of the extract itself as mentioned before in "the effect of temperature of clove extract on ΔE*" (figure 6.6). However, this factor was selected into the model for the compatible hierarchy since there was a significant interactive effect between temperature and pH as shown in table 5.5.

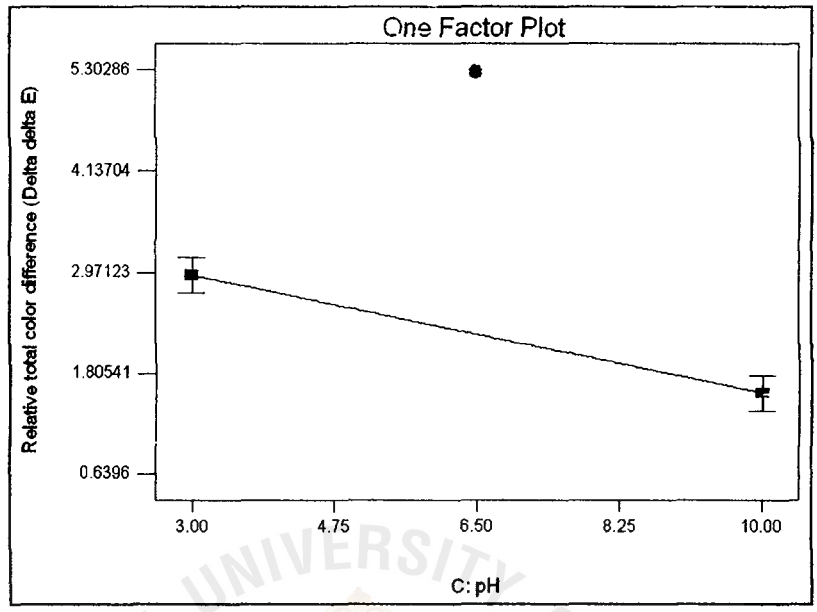

Figure 6.11: Effect of pH of clove extract on relative total color difference ($\Delta(\Delta E^*)$) of apple slices (center point (•) = treatment at 0.55% extract concentration, 25°C, pH 5.5).

Figure 6.11 showed that a significant decrease in $\Delta(\Delta E^*)$ was obviously observed with an increasing pH from 3 to 10. This can be implied that the clove extract at pH 3 provided more effective inhibition on the enzymatic browning than at pH 10. This was already mentioned earlier in "the effect of pH of clove extract on ΔE^*" (figure 6.7) that the inhibition of PPO activity are promoted at an acidic condition rather than alkaline condition [63]. Considering the effect of clove extract's pH on ΔE^* in figure 6.6, ΔE^* significantly increased at pH 10. This significant increase of ΔE^* (figure 6.6) may have contributed to the significant decrease in $\Delta(\Delta E^*)$ at pH 10 (figure 6.11), if the variation in $\Delta E_{\text{water control}}$-values is insignificant.

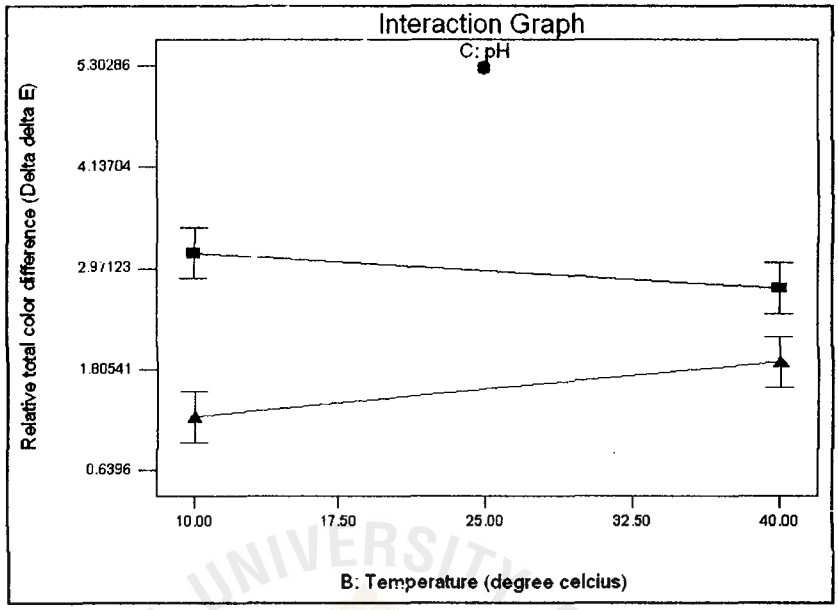

Figure 6.12: Interactive effect between temperature (B) and pH (C) (■ = pH 3, ▲ = pH 10) of clove extract on a relative total color difference ($\Delta(\Delta E^*)$) of apple slices. (center point (•) = treatment at 0.55% extract concentration, 25°C, pH 5.5).

Figure 6.12 showed that two lines in the plot were not parallel. This nonparallel lines implied that there was an interactive effect between temperature and pH of clove extract on the response $\Delta(\Delta E^*)$. From figure 6.12, as the temperature increased from 10°C to 40°C, $\Delta(\Delta E^*)$ remained constant at pH 3 (■), but significantly increased when pH was adjusted to 10 (▲). This implied that the effect of temperature on $\Delta(\Delta E^*)$ would be significant at only pH 10.

Considering the interactive effect of pH and temperature of the clove extract on ΔE^* in the figure 6.8, it did not correspond with the results of the response $\Delta(\Delta E^*)$ in figure 6.12. Generally, both ΔE^* and $\Delta(\Delta E^*)$ are negatively correlated. Since the results from figure 6.8 showed that ΔE^* at pH 3 significantly decreased at 40°C, $\Delta(\Delta E^*)$ at this pH in figure 6.12 was expected to significantly increase at 40°C. In addition, figure 6.8 showed that ΔE^* at pH 10 was constant at any temperature, so $\Delta(\Delta E^*)$ at pH 10 in figure 6.12 was also expected to come up with the same results. The non-correspondence between $\Delta(\Delta E^*)$ and ΔE^* from the interactive effect might be due to the variation in $\Delta E_{\text{water control}}$. The natural variation in phenolic substrate composition and PPO content in each apple can sometimes lead to a considerable variation of $\Delta E_{\text{water control}}$.

Consequently, this variation in $\Delta E_{\text{water control}}$ might cause $\Delta(\Delta E^*)$-value to deviate from the assumption as mentioned above.

By comparing $\Delta(\Delta E^*)$-value between pH 3 and 10 in figure 6.12, it was shown that, at any temperature, $\Delta(\Delta E^*)$ at pH 3 (■) still remained higher than that at pH 10 (▲). This implied that the clove extract at pH 3 could provide an inhibitory effect on the enzymatic browning greater than at pH 10. This is because the acidic condition has promoted the inhibitory effect of PPO inhibitors contained within the clove extract as mentioned earlier in "the effect of pH of clove extract on (ΔE^*)" (figure 6.7) [63].

Interpretation of the effects of the factors at the center point on the response ΔE^* and $\Delta(\Delta E^*)$.

Apart from the treatments created with two-factorial design, the treatment of both extracts was also applied at the middle level of three factors (extract concentration, temperature, and pH). This middle level can be called "center point". This treatment was performed at the condition of 0.55% extract concentration, 25°C and pH 5.5. The results of these treatments are represented with red (•) or green dots (•) in the plot of figure 6.1 to 6.12. Since these treatments were performed with a duplication, there were two dots shown in the plot.

According to a study of *Soysal (2009)*, the optimum temperature for apple PPO was reported as 25°C [91]. *McEvily, et al. (1992)* reported that apple PPO has the optimum pH range of 5-7 [63]. Considering the results of the treatments at center point of lemon balm extract from figure 6.1 to 6.4, the treatment at this condition provided the highest value of ΔE^* and lowest value of $\Delta(\Delta E^*)$. Since temperature at 25°C and pH 5.5 are within the optimum range for PPO activity, the treatment with lemon balm extract at this condition could promote the enzymatic browning rather than the inhibition. This might be a reason why the highest ΔE^*-value and the lowest $\Delta(\Delta E^*)$ were found when lemon balm extract at this condition has been applied.

Regarding to clove extract, the results of treatments at center point from figure 6.5 to 6.12 provided the lowest value of ΔE^* and highest value of $\Delta(\Delta E^*)$. However, since this condition is optimum for PPO activity, it is also expected to provide the results with the highest ΔE^* and

lowest $\Delta(\Delta E^*)$ like in the case of lemon balm extract. As clove extract contains a substantial amount of various chemical substances possessing antibrowning effect, the inhibitory effect of clove extract itself might be significantly greater than the promoting effect provided at the optimum condition. Besides, there is a variation of optimum pH and temperature of PPO. For instance, *McEvily, et al. (1992)* and *Underhill, et al. (1992)* reported that PPO are optimum at pH 5-7 and temperature at 25-40°C respectively [63, 98]. In conclusion, since clove extract provided a considerable inhibitory effect , and PPO's optimum pH and temperature could be varied due to a natural variation, the treatment of clove extract at the condition of center point might not always provide the highest ΔE^* and lowest $\Delta(\Delta E^*)$. These reasons could explain why the results in ΔE^* and $\Delta(\Delta E^*)$ obtained from clove extract at this condition had deviated from the assumption.

6.5 Interpretation of the results from optimization

After performing the factorial design to screen which factors significantly affected the inhibition of enzymatic browning expressed through the responses ΔE* and Δ(ΔE*), optimization was carried out to achieve the responses with the highest inhibitory effect. The combination of three factors (extract concentration, temperature, and pH) of each extract was optimized to a certain level that gave a minimum ΔE*-value and maximum Δ(ΔE*)-value.

The optimization was designated with predicted responses by the statistical software. Then, the optimized factor combination of each extract was selected based on the highest desirability. The predicted value of each response from selected combinations was then verified through experimentation. The results of the optimized clove and lemon balm extract application from figure 5.1 to 5.3 and figure 5.4 to 5.6 respectively, showed that the experimental ΔE* and Δ(ΔE*) corresponded with the predicted values. Considering the results from figure 5.1, 5.3, 5.4 and 5.6, it showed that the highest difference between the experimental and the predicted ΔE* was 0.52 (as shown in figure 5.4). This indicated that the proximity between the experimental ΔE* and the predicted ΔE* for both clove and lemon balm extract. The results from figure 5.2, 5.3, 5.5 and 5.6 showed that experimental Δ(ΔE*)-values of both clove and lemon balm extract application were positive, which coincided with the predicted values. This indicated the presence of inhibitory effect for all selected optimized combinations. In addition, the experimental and predicted Δ(ΔE*) values of both extracts were proximate to each other with the highest difference of 0.4 (as shown in figure 5.5). In conclusion, the small difference between the predicted and experimental values indicated that the statistical models are reliable and valid to use for explaining and predicting the outcomes of the experiment.

The deviations between the experimental and predicted values were not present in an unusual pattern. Also, these deviations were not especially found in the optimization of one extract or one response. Besides, these deviations are not considerable. The deviations could be caused by the natural variation in apple composition in term of PPO and phenolic compounds, and probably by the fluctuation of b*-value as mentioned in section 6.3. On the other hand, they might be resulted from the following causes. First, the PPO inactivation by pH is reversible, and the inhibitory effect of ascorbic acid in clove extract is temporary thus browning reaction can recover again before the end of storage [6]. For this reason, the experimental ΔE* could become higher than

the predicted one after storage. Second, since water core symptom, which is commonly found in apples, can result in the reduction of L*-value, the degree variation of this symptom in each apple could be postulated as one cause of the deviations.

According to the experimental results of the lemon balm extract optimization from figure 5.1 to 5.3, it can be summarized into the table 6.1.

Table 6.1: Experimental ΔE* and Δ(ΔE*) data obtained from optimization of lemon balm extract.

Lemon balm extract	**Optimized condition**			**Experimental Data**	
	Concentration	Temperature	pH	ΔE*	Δ(ΔE*)
Combination providing lowest ΔE*	0.10%	39.65°C	9.93	3.57	-
Combination providing highest Δ(ΔE*)	0.10%	38.47°C	5.08	-	1.61
Combination providing lowest ΔE* and highest Δ(ΔE*) simultaneously	0.10%	39.62°C	9.96	4.11	1.44

Note: The highlighted cells indicate the lowest ΔE*-value and the highest Δ(ΔE*)-value obtained from the optimization.

The experimental results of lemon balm optimization from table 6.1 showed that the condition at 0.1% concentration, 39.65°C and pH 9.93 provided the lowest ΔE* at 3.57. On the other side, the condition at 0.1% concentration, 38.47°C and pH 5.08 provided the highest Δ(ΔE*) at 1.61.

According to the experimental results of the clove extract optimization from figure 5.4 to 5.6, it can be summarized into the table 6.2.

Table 6.2: Experimental ΔE* and Δ(ΔE*) data obtained from optimization of clove extract.

Clove extract	Optimized condition			Experimental Data	
	Concentration	Temperature	pH	ΔE*	Δ(ΔE*)
Combination providing lowest ΔE*	1.00%	39.17°C	3.01	2.00	-
Combination providing highest Δ(ΔE*)	1.00%	10.00°C	3.01	-	4.27
Combination providing lowest ΔE* and highest Δ(ΔE*) simultaneously	1.00%	36.53°C	3.00	1.51	3.73

Note: The highlighted cells indicate the lowest ΔE*-value and the highest Δ(ΔE*)-value obtained from the optimization.

The experimental results of clove optimization from table 6.2 showed that the condition at 1% concentration, 36.53°C and pH 3.00 provided the lowest ΔE* at 1.51 . On the other hand, the condition at 1% concentration, 10.00°C and pH 3.00 provided the highest Δ(ΔE*) value at 4.27.

Normally, the condition providing the lowest ΔE* value is expected to provide the highest Δ(ΔE*) value, since the Δ(ΔE*) is negatively correlated with the ΔE* value. However, the results from table 6.1 and 6.2 showed that both conditions were not the same. This is because some factors did not have a significant effect on the responses. To illustrate, if the effect of temperature is not significant on Δ(ΔE*) response, this means that the highest Δ(ΔE*) can be obtained at any temperature which might not be the same with the condition that provide lowest ΔE*.

6.6 Comparison of the inhibitory effect of the optimized factor combination between clove and lemon balm extract on the enzymatic browning.

The experimental results of ΔE* and Δ(ΔE*) from the optimization of clove and lemon balm extract could be summarized into table 6.3.

Table 6.3 The experimental ΔE* and Δ(ΔE*) values from the optimization of clove and lemon balm extract application.

One response optimization (ΔE*)		
Extract	Clove	Lemon balm
ΔE*-value	2.00	3.57
One response optimization (Δ(ΔE*))		
Extract	Clove	Lemon balm
Δ(ΔE*) -value	4.27	1.61
Simultaneous two responses optimization (ΔE* and Δ(ΔE*))		
Extract	Clove	Lemon balm
ΔE*-value	1.51	4.11
Δ(ΔE*) -value	3.73	1.44

Table 6.3 showed that ΔE*-values from clove extract optimization were 1.51 and 2.0 which indicated fairly color changes [52]. But ΔE*-values from the lemon balm optimization were 3.57 and 4.11 which indicated perceptible color changes [52]. Regarding to Δ(ΔE*) response, table 6.3 showed that Δ(ΔE*) -values from clove extract optimization were 3.73 and 4.27. But Δ(ΔE*) -values from lemon balm optimization were 1.4 and 1.6. The results showed that the optimization of clove extract application provided a lower ΔE* and higher Δ(ΔE*) than those of lemon balm extract. The results from the factorial design from table 5.1 and 5.2 also corresponded with this outcome in which most treatments with clove extract showed a lower ΔE* and higher Δ(ΔE*) than those with lemon balm extract. This result indicated that the clove extract optimization provided a greater efficiency on the inhibition of the enzymatic browning

than the lemon balm extract optimization. The difference in the inhibitory strength is mainly due to the different type and amount of phenolic compounds contained within the extracts.

As mentioned before in section 6.4 , most phenolic compounds in clove extract would rather act as PPO inhibitors to inhibit the enzymatic browning reaction. On the other hand, lemon balm contains a higher amount of phenolic substrates which promote the browning reaction. However, lemon balm still contains some amount of phenolics inhibitors. Almost all of these phenolics inhibitors are competitive type in which they will be effective when they are present at the amount considerably higher than phenolic substrates [87]. Since the amount of phenolic inhibitors in lemon balm is likely lower than the substrates derived from the extract itself and apple, these inhibitors in lemon balm extract might not efficiently perform the inhibition on the browning [87]. Regarding the antioxidant activities, phenolic compounds can be postulated to inhibit the enzymatic browning based on these activities [103]. Since the antioxidant capacity of clove (346 μmol of trolox/100 g of dry weight) is higher than lemon balm (10.6 μmol of trolox/100 g of dry weight), this would be another explanation why the clove extract performed the inhibitory effect greater than lemon balm extract [104]. Although some of phenolic inhibitors present in both extracts belong to the same group, the inhibitory strength is different. To illustrate, flavonoids in clove extract have the inhibitory strength higher than that in lemon balm extract [16]. Moreover, the content of these compounds in clove is higher than that in lemon balm [20]. Both extracts contain benzoic acid which acts as PPO inhibitors, but the percentage of PPO inhibition of 2,4-dihydroxy benzoic in clove is higher than that of 3,4-dihydroxy benzoic in lemon balm [29]. The superiority of the inhibitory effect of clove over lemon balm is also attributed to the higher amount of ascorbic acid which efficiently inhibits the enzymatic browning [71]. Finally, it can be concluded that clove extract can inhibit the enzymatic browning more effective than lemon balm extract because clove extract has the higher amount of inhibitors and antioxidant activities, and the inhibitor type in clove extract provide a greater inhibitory strength.

6.7 Comparison the inhibitory effect on the enzymatic browning between sodium bisulfite, and the optimized factor combination from clove and lemon balm

As the final aim is to replace the use of sulfites on inhibiting the enzymatic browning in fresh-cut apples, apple slices were also treated with sodium bisulfite solution at 0.14% concentration and room temperature. This is a general condition industrially used by *Meyer Gemüsebearbeitung GmbH, Germany* who is the industrial fresh-cut fruits manufacturer and our project partner. However, the treatment with sodium bisulfite was executed without optimization. This treatment was carried out for the comparison of the inhibitory effect between sodium bisulfite and the optimized factor combination of the extracts.

From the results of the optimization of plant extracts (table 6.3), lemon balm extract can provide the lowest ΔE^* at 3.57 and the highest $\Delta(\Delta E^*)$ at 1.61. On the other hand, the optimization of clove extract can provide the lowest ΔE^* at 1.51 and the highest $\Delta(\Delta E^*)$ at 4.27.

The results from treatments with sodium bisulfite and the extracts are compared and shown in figure 6.13.

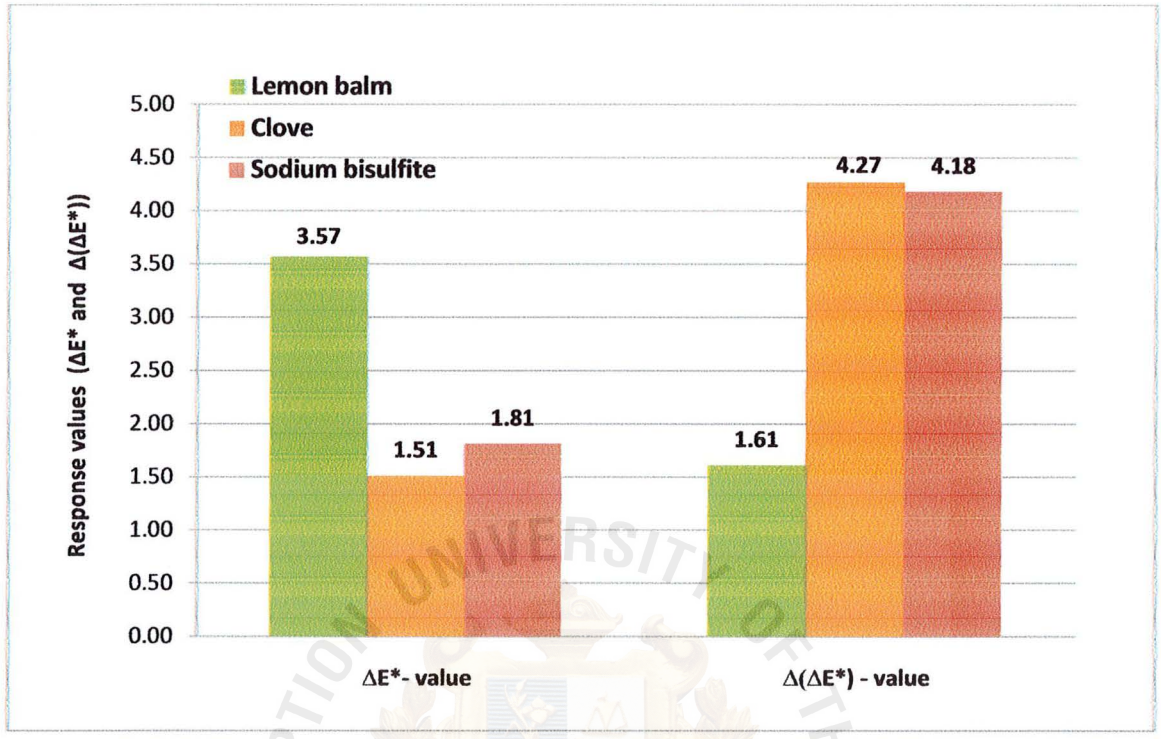

Figure 6.13: The experimental ΔE* and Δ(ΔE*) values from the optimization of the extracts (clove and lemon balm) and from the treatment with sodium bisulfite solution.

Note: The treatment with sodium bisufite was done without an optimization. It was applied with apple slices at industrial condition (0.14% concentration and room temperature)

Comparing the results between the treatment with lemon balm extract at the optimized condition and that with sodium bisulfite, figure 6.13 showed that ΔE*-value from sodium bisulfite (1.81) was lower than that from lemon balm extract (3.57). Regarding Δ(ΔE*)-value, the treatment with sodium bisulfite gave Δ(ΔE*)-value at 4.18 which was higher than that from lemon balm extract (1.61). The results of ΔE* and Δ(ΔE*) between lemon balm extract and sodium bisulfite implied that the inhibitory effect of sodium bisulfite on the enzymatic browning was greater than that of lemon balm extract. Apart from color measurement, the visual appearance was observed as well. Normally, if the results from color measurement indicated that which extract has the greater inhibitory effect on the enzymatic browning, the apple slices treated with that extract will be less brown than those treated by the other. The visual observation showed that apple slices treated

with lemon balm extract were obviously browner than those treated with sodium bisulfite by the end of storage. This showed that the result from visual observation confirmed those results from the color measurement. The lower inhibitory effect of lemon balm extract might be due to the reason that lemon balm extract contains the phenolic substrates at the higher amount than phenolic inhibitors [14, 104]. Besides, the action of phenolic inhibitors in the lemon balm extract is not effective as mentioned before in section 6.6. Although phenolic inhibitors in lemon balm extract such as flavonoids and tannin can perform the inhibition, they show only weak-to-moderate inhibitory effect [16]. Moreover, some phenolic inhibitors such as cinnamic acid and benzoic acid can be modified into PPO substrates to promote the enzymatic browning [85]. Even though lemon balm extract was applied at the optimized concentration, temperature and pH, the inhibitory effect of temperature and pH on the enzymatic browning might not be comparable to the promoting effect of phenolic substrates contained in lemon balm extract.

Comparing the results between the treatment with clove extract at optimized condition and that with sodium bisulfite, figure 6.13 showed that ΔE^*-value from sodium bisulfite (1.81) was higher than that from clove extract (1.51). Regarding $\Delta(\Delta E^*)$-value, the treatment with sodium bisulfite gave $\Delta(\Delta E^*)$-value at 4.18 which was lower than that from clove extract (4.27). The implication based on ΔE^* and $\Delta(\Delta E^*)$ indicated that the inhibitory effect of clove extract on the enzymatic browning was greater than sodium bisulfite. However, the results from visual observation showed that the apple slices treated with clove extract was browner than those treated with sodium bisulfite. This can be implied that the inhibitory effect of clove extract was still lower than sodium bisulfite. This result disagreed with those from the color measurement.

This contradiction between the results from color measurement and appearance observation could be resulted from some possible causes. Firstly, the nature of apple raw materials should be taken into account for a consideration. Each apple has a different degree of tissue sensitivities to the enzymatic browning so this would finally affect on ΔE^* and $\Delta(\Delta E^*)$ responses [66]. To clarify, apples ,which have the higher tissue sensitivities due to the higher PPO activities or substrate content, would tend to produce a higher value of ΔE^* and lower value $\Delta(\Delta E^*)$ than those which have the lower sensitivities. The difference in tissue sensitivities can be confirmed with the variation of $\Delta E_{\text{water control}}$ which is shown in appendix A (table 9.4). Secondly, there is a frequent report on the instability of b*-values which finally can contribute to the fluctuation of

ΔE^* and $\Delta(\Delta E^*)$ responses [22, 91]. The variation in b*-values is shown in appendix A (table 9.3). Lastly, the bleaching properties of sodium bisulfite should also be considered. Generally, the enzymatic browning causes a reduction of L*-value. However, instantaneous bleaching from sodium bisulfite might cause an apple flesh to be brighter so the L*-value is changed towards the positive direction [98]. This can be seen in the results of sulfite treatment in appendix B (table 9.7). Subsequently, an increase of L*-value has affected on ΔE^* and $\Delta(\Delta E^*)$ responses. These instances might be the causes of the false positive results of the color measurement. On the other point of view, the visual appearance might be a cause of misinterpretation. The bleaching effect from sodium bisulfite could be a cause to make apple slices treated with this substance to be less brown than those treated with clove extract although the degree of browning might not be significantly different. Moreover, since the color of clove extract is darker than sodium bisulfite, this might be a reason why the apple slices treated with clove extract were browner than those treated with sodium bisulfite.

Since there was a contradiction between the results from color measurement and visual appearance, the comparison results of the inhibitory effect on the enzymatic browning between sodium bisulfite and clove extract was still ambiguous. However, it can still imply that the inhibitory effect of clove extract were nearly comparable to sodium bisulfite since ΔE^* and $\Delta(\Delta E^*)$ results between those were proximate to each other. The promising results from the application of clove extract at optimized condition could be due to the reason that clove extract contains the various substances with a considerable inhibitory strength on the enzymatic browning. Moreover, the interactive affect between temperature and pH of clove extract had also contributed to the effective inhibition.

In conclusion, the use of lemon balm extract even at optimized condition did not provide the satisfied results on the inhibition of the enzymatic browning of fresh-cut apples since its inhibitory effect implied from ΔE^* and $\Delta(\Delta E^*)$ was considerably lower than clove extract and sodium bisulfite. Turning to clove extract, the color measurement results showed that the use of clove extract at optimized condition can provide the inhibition on the enzymatic browning better than sodium bisulfite. However, the potential of using the clove extract as sulfites substitutes is still ambiguous due to the contradiction between color measurement and actual visual appearance. Although the application of clove extract at optimized condition might not be able to

replace the use of sulfites, it can still perform the considerable inhibitory effect on the enzymatic browning. Therefore, the optimized factor combination of clove extract could be used as one alternative for the natural antibrowning agent.

6.8 Outlook

The parameter b* was frequently found to be unstable during the enzymatic browning [22, 91]. As the fluctuation of b* could further affect on ΔE* and Δ(ΔE*) response, I suggest to exclude b* parameter from the calculation of ΔE* and Δ(ΔE*) to reduce the deviation of these responses. Moreover, the bleaching effect of sulfites can cause an increase of L*-value which also contribute to the deviation of ΔE* and Δ(ΔE*). Therefore, ΔE* and Δ(ΔE*) might not be sufficient to express the inhibition of the enzymatic browning. Parameters such as ΔL* and Δa* that influences ΔE* and Δ(ΔE*) should also be taken into a consideration. Then, we can know whether the change of ΔE* and Δ(ΔE*) are really resulted from the effect of enzymatic browning or the other factors such as the bleaching effect from the sulfite treatment. This practice will provide more accuracy for the result interpretation and for the comparison of the inhibitory effect between extracts and sulfites. As the deviation of ΔE* and Δ(ΔE*) responses could also be resulted from the natural variation in the apples, we should find out the effective measures to control this variation.

Regarding to the results from color measurement, the application of clove extract at the optimized condition provided the promising results on the inhibition of enzymatic browning. But, its inhibitory effect based on visual appearance was not comparable to sodium bisulfite. Therefore, the application of clove extract as sulfite substitute was still uncertain. As the final aim is to replace the use of sulfite, the application of clove extract at optimized condition could be combined with the use of other antibrowning agents or physical methods to improve the inhibitory effect. The phenolic inhibitors and ascorbic acid in clove extract together with its pH and temperature effect could involve in the browning inhibition through antioxidant activity, reduction of *o*-quinones, and disruption of the PPO activity. This points out that the application of clove extract can inhibit the browning by affecting on PPO enzyme and intermediate products. Therefore, the methods, which can eliminate other essential components such as O_2 and phenolic

compounds, are suggested to use with clove extract to promote the inhibition. Nevertheless, the chemicals normally used to eliminate the phenolic substrates could also affect the phenolic inhibitors in the extract. Therefore, the method for removal of O_2 is more suitable and feasible. The method commonly used for oxygen exclusion is a modified atmosphere packaging and vacuum treatment. Therefore, the application of clove extract at optimized condition in conjunction with the modified atmosphere or vacuum packaging could be further investigated on the enzymatic browning inhibition for the substitution of sulfites.

In addition to the effective inhibitory effect, the sulfites alternative should also provide safety, cost-effectiveness, and not affect on a sensory quality as mentioned in section 3.3.2. Thus, these three issues must be assessed. Since the plant extracts are derived from natural sources and normally used as a flavoring agent in the food, it could be safe to use them as antibrowning agents in fresh-cut apples. However, it is necessary to determine and consider the acceptable daily intake level of the extract. On the other hand, the application of extracts at certain concentration, pH and temperature can cause the undesirable change in sensory properties of fresh-cut apples in term of color, texture and flavor [81]. Therefore, the comprehensive sensory and consumer tests should be further performed to determine the effect of the extract application on the sensory quality of apple slices and the consumer acceptance. Lastly, the cost-effectiveness for the extraction process and the application of extracts should be evaluated and determined.

7. Conclusion

The aim of this work is to optimize an application of clove and lemon balm extract to substitute sulfur dioxide or sulfites for inhibiting the enzymatic browning of fresh-cut apples. The application took the concentration, temperature and pH of the extracts into account. The effect of these three factors on the inhibition of enzymatic browning was investigated by two-level factorial design. The degree of inhibition was determined in term of color change expressed through ΔE^* and $\Delta(\Delta E^*)$ responses in which a lower ΔE^* and higher $\Delta(\Delta E^*)$ implies the greater inhibition ability. Considering lemon balm extract, all of three factors had a significant effect on ΔE^* in which ΔE^*-value was decreased at lower concentration, but at higher temperature and pH. On the other hand, only extract concentration had a significant effect on $\Delta(\Delta E^*)$ in which $\Delta(\Delta E^*)$-value dropped at a lower concentration. There was no evidence of any interactive effect for lemon balm extract application.

Regarding to the application of clove extract, all of three factors excepting temperature had a significant effect on both ΔE^* and $\Delta(\Delta E^*)$. The lower ΔE^* and higher $\Delta(\Delta E^*)$ values were obtained at an increased extract concentration but decreased pH. Moreover, there was a significant interactive effect between temperature and pH of the clove extract on both responses. The interactive results showed that the lowest ΔE^* and highest $\Delta(\Delta E^*)$ values were obtained when clove extract was adjusted to 40°C combined with pH 3.

After performing two-level factorial, the optimization was carried out to find out the optimum condition of the application of clove and lemon balm extract to achieve the highest inhibitory effect on the enzymatic browning. The optimized design proposed that the application of lemon balm extract at 0.10% concentration, 39.62 °C and pH 9.96 could provide the highest inhibition with simultaneous minimum ΔE^* and maximum $\Delta(\Delta E^*)$. On the other hand, the application of clove extract at 1% concentration, 36.53 °C and pH 3.00 simultaneously gave the minimum ΔE^* and maximum $\Delta(\Delta E^*)$. Then, the predicted results from optimization were verified through experimentation. The experimental results were proximate to the prediction. This propinquity indicated the reliability of the model.

A comparison between the application of clove and lemon balm extract at the optimized condition showed that clove extract could provide a greater inhibitory effect than lemon balm

extract. Comparing with sodium bisulfite based on the color measurement, the inhibitory effect of lemon balm extract at optimized condition was less than that of sodium bisulfite. On the other hand, the optimized clove extract application provided inhibition was greater than sodium bisulfites. However, the visual appearance exhibited the contradiction. It showed that the application of clove extract still provided a lower inhibitory effect than that of sodium sulfite. This contradiction indicated that the comparison on the inhibitory effect between clove extract and sulfites was ambiguous. Nevertheless, the application of clove extract at optimized condition still provided the promising results with the considerable inhibition on the enzymatic browning. The inhibition could be resulted from the phenolic inhibitors and ascorbic acid contained in the clove extract. Moreover, the inhibition could be supported by the effect of temperature and pH at the optimized condition, which help to retard the PPO activity. Therefore, the optimized factor combination of clove extract could be considered as one alternative for natural antibrowning agents.

8. References

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9. Appendix

APPENDIX A: Color measurement results from the application of plant extracts: clove and lemon balm.

According to the two-level full factorial design, the results from the color measurement of the apple slices treated with the extracts are shown in the table 9.1 and 9.2. The results are provided with ΔL^*, Δa^*, Δb^*, ΔE^*, $\Delta E_{control}$, and $\Delta(\Delta E^*)$ values.

The results from the color measurement of the apple slices treated with clove extract and water as control from run no. 1 to no. 10 are shown in the table 9.1.

Table 9.1: The results from the color measurement of the apple slices treated with clove extract.

Run	ΔL^*	Δa^*	Δb^*	ΔE^*	$\Delta E_{water\ control}$	$\Delta(\Delta E^*)$
1	-0.78	1.22	2.35	2.76	4.95	2.19
2	-0.72	1.60	2.08	2.72	5.48	1.88
3	-2.57	3.21	2.89	5.02	7.36	2.33
4	-0.98	1.94	-0.39	2.21	3.09	5.30
5	-1.07	2.09	0.95	2.54	7.79	5.25
6	-3.75	3.08	-0.61	4.89	7.72	2.83
7	-1.26	2.76	4.50	5.43	6.07	0.64
8	-0.12	1.50	0.38	1.55	4.85	3.29
9	-1.97	3.07	4.65	5.91	6.92	0.96
10	-0.72	1.60	2.08	2.72	6.68	3.96

Note: $\Delta E^* = \sqrt{(\Delta L^*)^2 + (\Delta a^*)^2 + (\Delta b^*)^2}$

$\Delta(\Delta E^*) = \Delta E_{water\ control} - \Delta E^*$

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The results from the color measurement of the apple slices treated with lemon balm extract and water as control from run no. 1 to no. 10 are shown in the table 9.2.

Table 9.2: The results from the color measurement of the apple slices treated with lemon balm extract.

Run	ΔL*	Δa*	Δb*	ΔE*	$\Delta E_{water\ control}$	Δ(ΔE*)
1	-2.19	3.17	5.49	6.71	9.06	2.35
2	-3.10	7.49	6.89	10.64	4.33	-6.31
3	-3.82	4.30	8.37	10.16	11.00	0.85
4	-5.49	6.72	12.54	15.25	9.24	-6.01
5	-6.24	6.57	12.83	15.70	10.02	-5.68
6	-4.88	6.56	1.75	8.36	3.10	-5.27
7	-1.89	3.33	4.87	6.20	8.13	1.94
8	-7.65	6.72	2.95	10.60	5.38	-5.22
9	-1.09	3.19	2.07	3.95	5.28	1.32
10	-7.14	9.39	7.78	14.13	7.47	-6.66

The results of the Δb* variation of the apple slices treated with clove and lemon balm extracts shown are in the table 9.3.

Table 9.3: The results of Δb* in ascending order of the apple slices treated with clove and lemon balm extract.

Clove extract application		Lemon balm extract application	
Run	Δb*	Run	Δb*
6	-0.61	6	1.75
4	-0.39	9	2.07
8	0.38	8	2.95
5	0.95	7	4.87
2	2.08	1	5.49
10	2.08	2	6.89
1	2.35	10	7.78
3	2.89	3	8.37
7	4.5	4	12.54
9	4.65	5	12.83
Range	-0.61 – 12.83		
Mean	4.22		
S.D.	3.84		

The results of the $\Delta E_{water\ control}$ variation of the apple slices treated with water as control in the application of both clove and lemon balm extract shown are in the table 9.4.

Table 9.4: The results of $\Delta E_{water\ control}$ in ascending order of the apple slices treated with water as control in the application of both clove and lemon balm extract.

Clove extract application		Lemon balm extract application	
Run	**$\Delta E_{water\ control}$**	**Run**	**$\Delta E_{water\ control}$**
4	3.09	6	3.1
8	4.85	2	4.33
1	4.95	9	5.28
2	5.48	8	5.38
7	6.07	10	7.47
10	6.68	7	8.13
9	6.92	1	9.06
3	7.36	4	9.24
6	7.72	5	10.02
5	7.79	3	11
Range	3.09 – 11.00		
Mean	6.48		
S.D.	2.18		

According to the optimization, the results from the color measurement of the apple slices treated with the extracts are shown in the table 9.4 and 9.5. The results are provided with ΔL^*, Δa^*, Δb^*, ΔE^*, $\Delta E_{control}$, and $\Delta(\Delta E^*)$ values.

The results of color measurement from the optimization of the apple slices treated with clove extract are shown in the table 9.5.

Table 9.5: The results of color measurement from the optimization of the apple slices treated with clove extract.

Optimization	ΔL^*	Δa^*	Δb^*	ΔE^*	$\Delta E_{water\ control}$	$\Delta(\Delta E^*)$
One response optimization (ΔE^*)	0.07	1.95	0.46	2.00	4.22	2.22
One response optimization ($\Delta(\Delta E^*)$)	-0.17	1.03	-0.86	1.35	5.63	4.27
Simultaneous two responses optimization (ΔE^* and $\Delta(\Delta E^*)$)	-0.25	1.38	-0.62	1.51	5.25	3.74

The results of color measurement from the optimization of the apple slices treated with lemon balm extract are shown in the table 9.6.

Table 9.6: The results of color measurement from the optimization of the apple slices treated with lemon balm extract.

Optimization	ΔL*	Δa*	Δb*	ΔE*	$\Delta E_{water\ control}$	Δ(ΔE*)
One response optimization (ΔE*)	-1.58	2.23	2.31	3.57	3.61	0.04
One response optimization (Δ(ΔE*))	-1.42	2.39	1.82	3.32	4.93	1.61
Simultaneous two responses optimization (ΔE* and Δ(ΔE*))	-2.02	2.19	2.83	4.11	5.55	1.44

APPENDIX B: Color measurement results from the application of sodium bisulfite.

The results from the color measurement of the apple slices treated with sodium bisulfite at 0.14% and room temperature are shown in the table 9.7 The results are provided with L*, a*, b*, ΔL*, Δa*, Δb*, ΔE*, $\Delta E_{control}$, and Δ(ΔE*) values.

Table 9.7: The results of color measurement of the apple slices treated with sodium bisulfite.

Measurement time (minutes)	L*	a*	b*	ΔL*	Δa*	Δb*	ΔE*	$\Delta E_{water\ control}$	Δ(ΔE*)
0	82.20	-4.47	25.61	-	-	-	-		
5	82.42	-4.48	25.73	0.21	-0.01	0.13	0.25		
10	82.48	-4.56	25.87	0.28	-0.08	0.26	0.39		
15	81.85	-4.63	26.17	-0.35	-0.15	0.56	0.68		
20	82.51	-4.63	25.98	0.30	-0.15	0.38	0.51		
25	82.48	-4.61	26.01	0.28	-0.14	0.40	0.51		
30	82.49	-4.65	26.10	0.29	-0.18	0.49	0.59		
35	82.57	-4.69	26.35	0.37	-0.21	0.75	0.86		
40	82.64	-4.62	26.18	0.44	-0.15	0.57	0.74		
45	82.71	-4.60	26.13	0.51	-0.12	0.53	0.74		
50	82.85	-4.60	26.16	0.65	-0.13	0.55	0.86		
60	82.75	-4.72	26.39	0.55	-0.25	0.78	0.99	5.99	4.18
70	82.64	-4.71	26.57	0.43	-0.24	0.96	1.08		
80	82.62	-4.80	26.71	0.42	-0.33	1.10	1.22		
90	82.71	-4.81	26.78	0.51	-0.34	1.18	1.33		
100	82.77	-4.77	26.72	0.57	-0.30	1.12	1.29		
110	82.78	-4.85	26.80	0.57	-0.38	1.19	1.37		
130	82.63	-4.79	26.82	0.42	-0.32	1.21	1.32		
150	83.14	-4.83	26.73	0.94	-0.36	1.12	1.51		
170	82.88	-4.82	26.65	0.67	-0.34	1.04	1.29		
190	83.00	-4.87	26.96	0.79	-0.40	1.35	1.62		
210	83.30	-4.85	26.95	1.10	-0.38	1.35	1.78		
230	83.48	-4.93	26.81	1.28	-0.45	1.20	1.81		

Note: The selected ΔE*-value is 1.81 as highlighted.